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Trauma to the head can result in the loss of teeth, injury to the ear and fracture of the jaw and/or cranium. The ability to produce materials and/or devices that can be used easily and effectively in the treatment of maxillofacial trauma is greatly needed. In addition, the ability to combine therapeutic agents with the restorative materials may greatly enhance the healing process or reduce the possibility of subsequent infection. Near net shape forming (NNSF) is a unique method for producing ceramic components which have high strength in the green state. Therefore, the aim of this proposal is to investigate the use of near net shape forming as a potential method for quickly fabricating both temporary and permanent maxillofacial restorative materials and/or devices possessing a wide range of physical characteristics. To meet these objectives, two types of non-toxic, thermally and chemically activated, gellable binders were investigated. Thermally activated gels become rigid when heated or cooled to a temperature that activates gelation. Chemically activated gels cross-link in the presence of various cations. Binders investigated include chitosan, xantham gum, locust bean gum and carageenan. These were mixed with either hydroxyapatite or tricalcium phosphate powders and the resulting composites were evaluated for chemical, biological, and mechanical properties. Formulations were produced that enable the composites to be injected through a syringe. Results indicate that composite mechanical properties are controlled by the amount of powder present, and the morphology of the crystals in the powder. Additionally, results indicate the it is possible to produce material that is porous, can release therapeutic agents to the would site, and are biodegradable. Unfortunately, the initial in vivo work indicated that the binders can cause a localized inflammatory response and may not be suitable. However, the concept of a physiologically stimulated polymer/ceramic composite shows promise given a suitable binder system.

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1. INTRODUCTION

Currently, large bone defects are repaired by using three general approaches. Autografts, where the donor and recipient are the same, have several disadvantages including increased operative times, post-operative complications, of limited supply, high morbidity rates, and donor site pain. Complications associated with this procedure have been reported to be as high as 20% (1). Another approach, allograft, where donor and recipient are genetically dissimilar, has limitations including graft rejection and the concern over disease transmission from donor to recipient. The third approach, synthetic grafts, which include materials such as metals, plastics, ceramics and composites thereof, has issues of biocompatibility of the graft and dissimilar mechanical behavior. Ideally bone replacement materials should be biologically compatible, should mimic the natural tissue in size, morphology, consistency and function, should not be predisposed to infection, does not evoke a healing response that alters its material characteristics, will be replaced by natural tissue, can be tolerated permanently, has an elastic modulus equal to that of bone, and has a higher fracture toughness than that of the natural bone so that mechanical failure will occur within the natural bone than in the replacement

For the treatment of bone loss due to trauma or disease, bioceramic materials have been used as artificial bone fillers (1). Specifically, the bioceramics hydroxyapatite (HAP) and tricalcium phosphate (TCP) have enjoyed wide use as bone replacements in the fields of oral and plastic surgery. HAP is classified as a bioactive ceramic because it can develop chemical bonds to the surrounding tissue and facilitates the attachment of the implant to these tissues. TCP, on the other hand, is classified as a resorbable bioceramic because it is gradually resorbed or dissolved and slowly replaced by the natural tissue. In dentistry, the main application has been in the filling of pockets and the augmentation of deficient mandibular or maxillar ridges caused by the loss of dentition due to advancing age, trauma, or congenial defect. Surgeons have also used these materials to contour traumatic or congenital defects in the bony arches of the face and cranium.

The diversity of bone replacement applications requires a variety of implant structures including porous, nonporous, pre-formed, granular, and/or paste forms. As with the composition of the replacement material, the architecture of the device can be very important to implant performance. Studies have shown that in low-load or non-load bearing applications, nearly inert porous ceramics can provide the necessary structure and scaffold for new bone formation because they provide a mechanism for capillary

ingrowth and tissue regeneration (2-3). In order for scaffolds to function *in-vivo*, they must allow cells to attach, migrate, differentiate, and proliferate. While the choice of material greatly affects these processes, the structure will also have dramatic affects on cellular response as well. Pore size and structure greatly influence the ability of cells to remain viable, provide the conduits for nutrients to reach the cells, and provide the template for cells to organize into tissues.

Unfortunately, the manufacturing process for these implants varies depending upon the type of material and/or the desired structure (porous, nonporous, resorbable, etc...). Typically, high pressure powder compaction is used to fabricate total joint components, such as femoral heads. Another method is hot isostatic pressing where the ceramic powder is compressed from all directions while sintering. Porous materials, for applications including bone scaffolds, are made by homogenizing the ceramic material with different organic substances which are subsequently removed thermally or via hydrothermal exchange processes. A major disadvantage of these techniques is that the material and/or device must be machined and/or fitted to final part dimensions. Typically, just the added step of machining can double the cost of manufacturing.

The use of a net shape fabrication process may address many of these shortcomings, while providing a faster simpler way of producing both pre-formed and quick setting restorative devices. The Pacific Northwest National Laboratory has developed a net shape fabrication process, "Near Net Shape Forming (NNSF)". NNSF utilizes cross-linkable binder systems which are mixed with a ceramic powder. The binder systems are a diverse family of materials, such as the polysaccharides, and can be selected to fit specific processing and material needs. The ceramics can be alumina or transformation toughened zirconia for femoral head replacements or hydroxyapatite for porous bone scaffolds. The ceramic-binder mixture is formed and subsequently cross-linked resulting in the formation of a rigid network of binder and ceramic material. Cross-linking may be controlled either by pH, ionic strength, temperature, and/or concentration. The as-formed part may then be sintered to produce a densified part. A unique feature of the NNSF process is that the as-formed material has structural integrity which is a result of the cross-linking. Thus, it can be mixed into a paste-like consistency, implanted, and hardened physiologically within a narrow time frame, ranging from minutes to hours. Developing injectable bone pastes that can fill large bone defects and harden under physiological conditions addresses the need for injectable, biocompatible, calcium phosphate composite materials.

The proposed work investigated the feasibility of using NNSF as a process for producing pre-formed and quick setting bone implant materials and devices. Pre-formed devices such as dental roots and various blocks for bone fillers can be a sintered, dense component and/or porous pre-formed device made from tricalcium phosphate and/or hydroxyapatite. Quick setting fillers take advantage of stimuli sensitive binders to fabricate a biocompatible binder/ceramic paste that hardens at physiological conditions.

To meet these objectives, two types of non-toxic, thermally and chemically activated, gellable binders were investigated. Thermally activated gels become rigid when heated or cooled to a temperature that activates gelation. Chemically activated gels cross-link in the presence of various cations.

For the performance period, the project was broken down into tasks; Binder Development and Rheological Optimization (Task 1), Component Fabrication (Task 2), Characterization of Materials Properties (Task 3), Biological Response (Task 4), and Development of Resorbable Tricalcium Phosphate/Chitosan Composites for Bone Tissue Replacement (Task 5).

2. BODY

2.1 Binder Development and Rheological Optimization

2.1.1 Materials and Methods

The polysaccharides and their commercial sources that were evaluated during this task were xanthan gum (Kelco), locust bean gum (Aldrich Chemical Company, Inc), and carrageenan (Aldrich Chemical Company, Inc). Xanthan gum solutions were prepared by addition of the xanthan gum powder into deionized water or sodium chloride solution with the desired ionic strength by mechanical stirring at room temperature. The resulting smooth solution was then allowed to sit overnight to remove entrapped air bubbles. Solutions of locust beam gum and carrageenan gum were prepared at 70-75°C and mechanically stirred until all powders dissolved.

Hydroxyapatite(HAP)-xanthan gum and HAP-locust bean gum pastes were prepared by mixing the desired amount of HAP powder (Aldrich) into the polysaccharide solution by using either a magnetic stir plate at solids loadings less than 30 weight percent (wt%) or by a mechanical stirrer at higher solids loadings greater than 30 wt%. To prepare HAP-carrageenan paste, the carrageenan solution was heated

to and maintained in a 70-75°C water bath during mixing. Rheological measurements, such as viscometry, were conducted using a Bohlin Rheometer.

2.1.2 Results and Discussion

2.1.2.1 Binder Selection/Development

The initial effort was focused on the selection of potential polysaccharide binders for use in both fabrication and bone filler materials. The polysaccharide binders were initially selected as candidate binder systems based on their chemical and physical properties as well as available oral toxicity data. Specifically, polysaccharides were chosen because they were non-toxic and could be easily handled in laboratory and field environments. In addition, polysaccharides are widely available. Due to the basic chemical structure, polysaccharides are typically water soluble, gellable by a variety of stimuli, and exhibit shear thinning behavior. Molecular weight, purity and functional groups can be tailored to control rheological and chemical properties. Due to the numerous polysaccharides available from plant and microbial sources, a screening process was used to eliminate polysaccharides that were skin sensitizers and allergens, elicited an immune response, and/or had an incompatibility with physiological conditions and high cost (in the case of fabrication binder systems). The three initial binders identified were xanthan gum, locust bean gum and carrageenan.

Xanthan Gum

Xanthan gum is a high molecular weight natural carbohydrate which is produced via fermentation by the microorganism, *Xanthomonas campestris*. Xanthan was initially identified because of its rheological properties. Xanthan gum exhibits a high viscosity at low polymer concentrations and at low shear rates. In the bone paste application, a high viscosity is desirable to maintain structural integrity once injected into the bone tissue. In the fabrication application, low polymer concentrations are necessary to limit organic content and ensure complete binder burn-out and densification. Xanthan also exhibits a high degree of pseudoplasticity and a high elastic modulus. When shear stress is increased on a ceramic-xanthan gum solution, viscosity is progressively reduced. When shear is reduced or remove, the starting viscosity is almost instantaneously recovered. This behavior is necessary in a ceramic-polymer system because flow is required during fabrication to ensure complete mold fill and during bone paste replacement to provide an injectable composition and complete tissue cavity replacement. Xanthan gums

are also stable or compatible with heat, pH, shear, enzymes, and chemicals, such as, salts, acids, and bases.

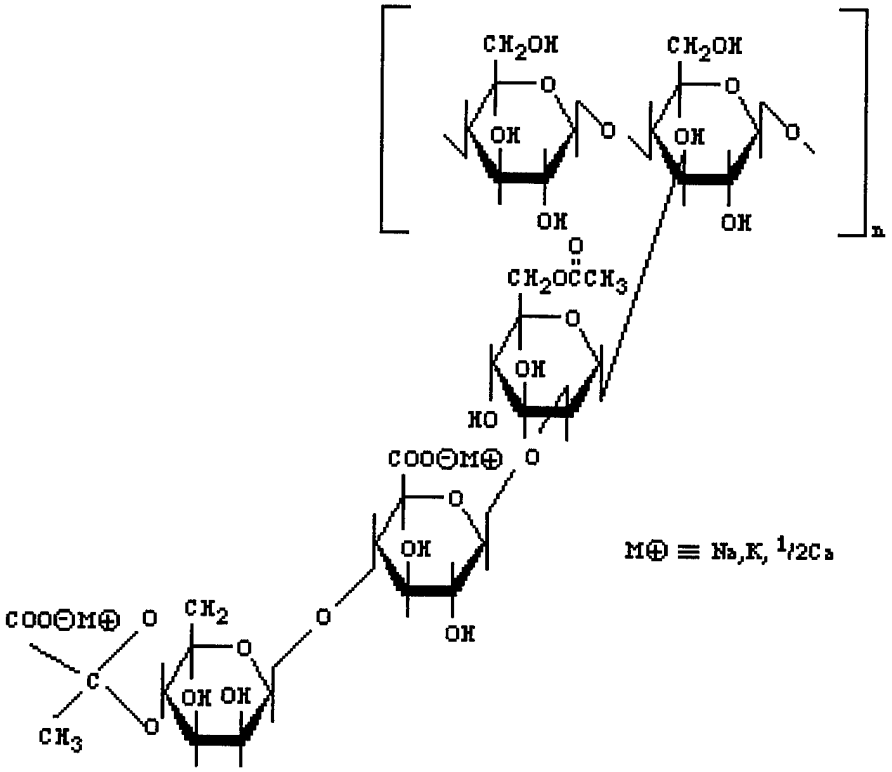

Figure 1. Structure of Xanthan Gum (4, 5).

These properties of xanthan gum can be related to its structure, shown in Figures 1 and 2 (4 - 5). Xanthan gum's repeat unit, shown in Figure 1, consists of five sugar residues: two glucose, two mannose, and one glucuronic acid. The helical conformation, which is shown in Figure 2, is stabilized by trisaccharide side chains which align with the polymer backbone through noncovalent interactions, principally hydrogen bonding. The high viscosity seen at low shear rates is a result of this highly ordered, stiff network. It is proposed that the shear thinning behavior results from disaggregation of this network and alignment of individual polymer chains in the direction of shear force (5). However, when shear is removed, the aggregates reform causes viscosity to increase again. The helical structure is also responsible for xanthan's viscoelastic insensitivity to temperature, ionic strength and pH. Other polysaccharides which are polyelectrolytes and have a less ordered or "random" coil conformation will be sensitive to changing electrolyte levels causing a dramatic change in solution viscosities. Understanding the polysaccharide structure and it's effect upon viscoelastic changes was subsequently important when

selecting the appropriate polymer binder for the proposed bone replacement applications, i.e., fabrication or paste, which will have separate rheological needs.

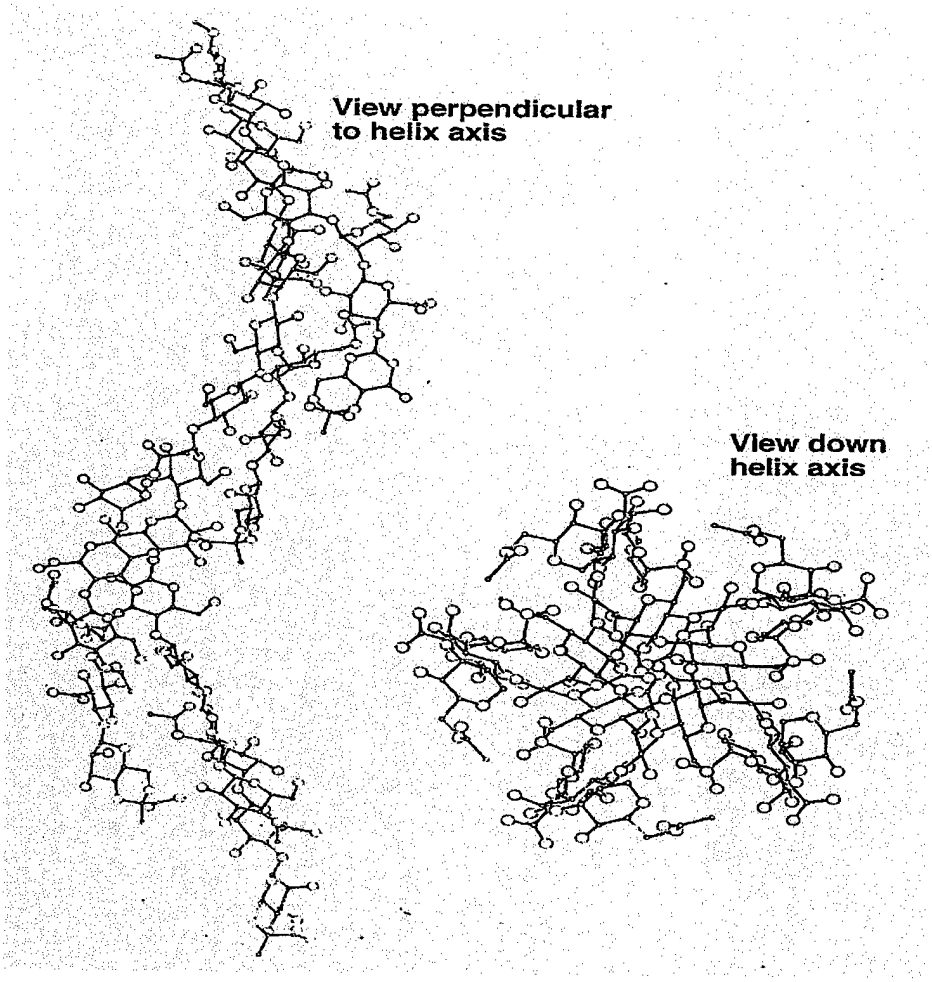

Figure 2. Helical Conformation of Xanthan Gum Viewed Parallel and Perpendicular to Helix Axis (5).

Locust Bean Gum

Locust bean gum is a carbohydrate polymer like xanthan gum containing galactose and mannose in the base repeat unit (4). However, locust bean is a linear chained polymer with nonuniformly spaced side chains resembling more of a natural block polymer. Locust bean gum has limited water solubility requiring heating to high temperatures (80°C) to reach full hydration. Upon cooling, locust bean will remain in solution but in a dissociated and extended form which is what initially identified it as a good thickening agent or binder for the proposed applications. Locust bean gum solutions contain endosperm which must be filtered when the application is for bone paste. Solutions of locust bean gum are pseudoplastic like xanthan with viscosity decreasing at very low shear rates. Locust bean gum solutions,

however, will degrade irreversibly with high shear rates. The degraded solutions will tend toward a Newtonian behavior with viscosity becoming constant with increasing shear.

Carrageenan

Carrageenans, derivatives of certain red seaweed species, are chemically sulfated linear polysaccharides (4). The half-ester sulfate groups are what define carrageenans to be anionic polyelectrolytes. The charged nature of the sugar units and their structural arrangement within the polymer macromolecule is what accounts for the carrageenans being highly reactive chemically and for the physical properties, such as the ability to form gels.

All carrageenans have the common structural feature of being linear polysaccharides built up by alternating 1,3-linked β-D-galactopyranosyl and 1,4-linked α-D-galactopyranosyl units (4). There are numerous possibilities for substitution on the basic copolymer thus resulting in a wide variety of carrageenan types. However, these types exist in variations or hybrids of seven "ideal" polysaccharides of definite chemical structure; *mu-*, *kappa-*, *nu-*, *iota-*, *lambda-*, *theta-*, and *xi-* carrageenans. Only *kappa-*, *iota-*, and *lambda-* carrageenans have commercially available sources. Of the three hybrid carrageenans, only *kappa-* and *iota-* are gelling species.

The chemical reactivity of the carrageenans is partly due to the half-ester sulfate groups ($R\text{-}O\text{-}SO^{3-}$) which are strongly anionic. The free acid group is unstable. The commercial carrageenans are available as stable sodium, potassium, or calcium salts, or more commonly, as mixtures of these. The associated cations with the conformation of the sugar units determine the physical properties of the polymer. For example, *kappa-* and *iota-* carrageenans gel most strongly with K^+ and Ca^{2+}, respectively.

The carrageenans are soluble in hot water (>70°C), and solubility is only limited by the viscosity of the solution. Due to the unbranched, linear structure and the polyelectrolytic nature of the carrageenans, they form highly viscous solutions. The mutual repulsion of the negatively charged half-ester sulfate groups along the polymer chain causes it to become rigid and highly extended while the hydrophilic nature causes the molecule to be surrounded by a sheath of immobilized water molecules (4). This creates a resistance to flow which can be altered by concentration, temperature, solutes, and molecular weight. For example, the viscosity of carrageenan solutions will decrease with increasing temperatures. *Kappa-* and *iota-*

carrageenans are able to form gels upon the cooling from a hot solution and are thermally reversible, i.e., they melt on heating and gel again upon cooling. As reported by Rees, aqueous carrageenan gels as a result of the double-helix formation (6). At elevated temperatures, thermal agitation overcomes the tendency to form helices, and the polymer exists as a random coil in solution. On cooling, however, a three dimensional polymer network builds up forming the junction zones of the polymer chains . Further cooling leads to the aggregation of these junction zones.

Iota- carrageenans do not form gels when it is in the sodium form. It was in question whether *iota-* carrageenans would gel in the presence of excess sodium, which is present in physiological body solutions. As a result, *iota-* carrageenan was eliminated from the feasibility list.

Locust bean gum is frequently used with k-carrageenan to modify carrageenan gel strength. Locust bean gum has been shown to bind with the helixes in the carrageenan gel, thus providing additional cross-linkages which will enhance gel strength and compliance. *Iota-* carrageenans do not show any interaction with locust bean gum.

The viscosity and gelation of these binders were studied and compared as discussed below.

2.1.2.2 Rheological Studies of Calcium Phosphate-Binder Suspensions

The viscosity of HAP suspensions containing one of the three binders selected was measured as a function of binder properties, HAP solids loading and temperature. The effect of calcium phosphate phase and/or morphology was also investigated by comparison of HAP and β-tricalcium phosphate (β-TCP).

Viscosity is a transport property. It must be sufficiently low to facilitate suspension transfer to the mold. Shear thinning behavior was desired. This means at very low shear rates suspended HAP particles remain stationary because the high viscosity of binder solutions below the yield point. Higher shear rates that are encountered during pouring, injecting or on a vibration bed during filling can effectively reduce the viscosity. As shown in Figure 3, all three slurries containing 30 wt% HAP showed shear thinning behavior. Among them, HAP suspension containing carrageenan has the lowest viscosity even though the carrageenan concentration is higher (0.4 wt%) then the other two binders (0.25 wt%).

Solids loadings had a great influence on viscosity (Figure 4). The viscosity of these systems was almost constant with HAP solids loading equal or below 30 wt% (Figure 5). Further increasing the solids loading to 40 wt%, dramatically increased the viscosity of the suspensions. The highest HAP loading that was achieved with these polymer systems was approximately 43 wt%. Above 43 wt%, it became difficult to measure its rheological property and to fabricate components without creating fill defects.

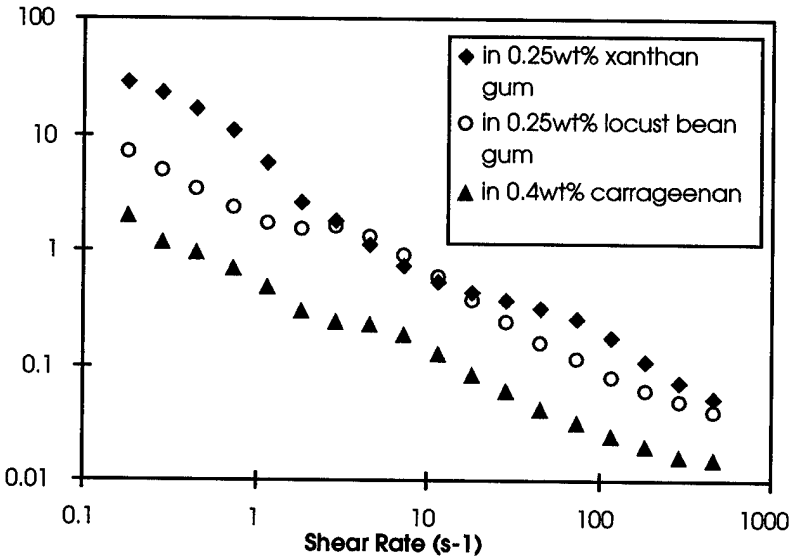

Figure 3. Viscosity of HAP Slurries with Different Binders.

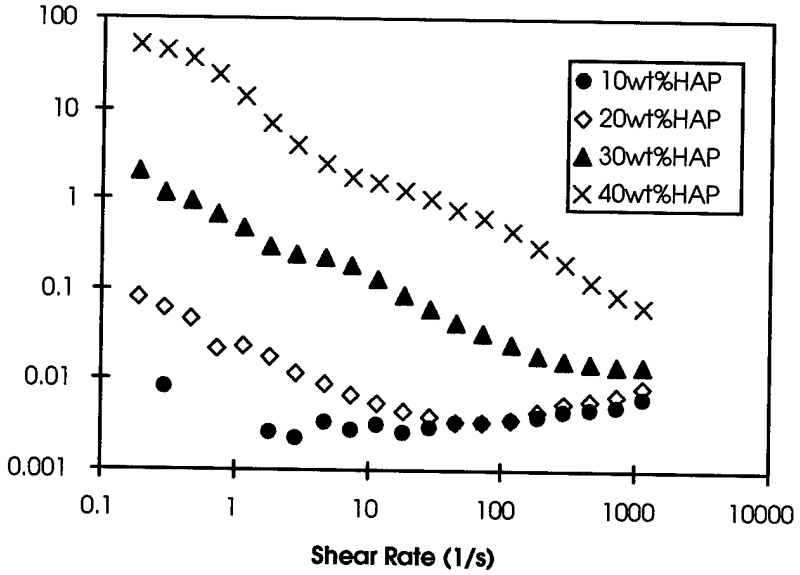

Figure 4. Viscosity of HAP in 0.4 wt% Carrageenan.

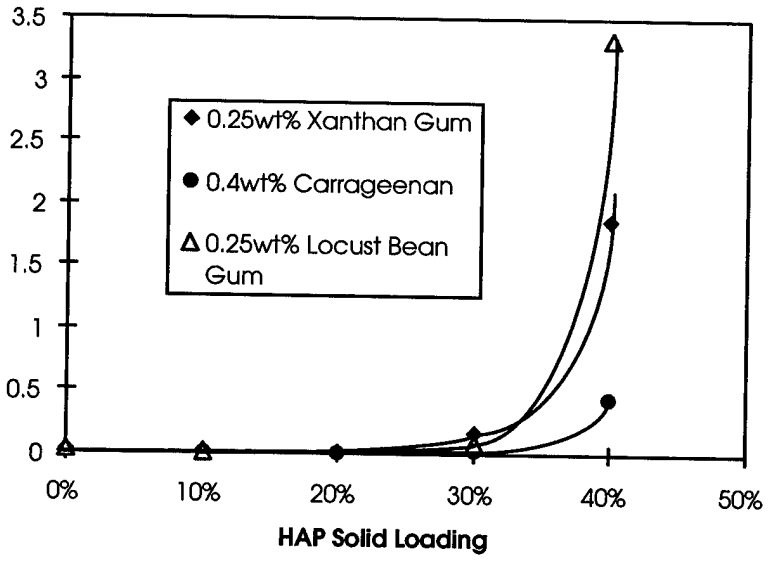

Figure 5. Viscosity of HAP-Polysaccharide Suspension at Shear Rate of $116s^{-1}$ as a Function of Solids Loading.

Effect of temperature on gelation properties was also investigated. It is known that temperatures up to 93°C have no effect on viscosity of xanthan gum solution. Locust bean gum solution, prepared at 70-80°C, also showed no significant changes in viscosity as temperature decreases. *K*-carrageenan solution, prepared at 70-75°C in a similar way to locust bean gum, formed a rigid gel upon cooling. The rheological behavior was investigated as a function of temperature. Temperature dependent viscosity measurements of carrageenan were started at 70°C and eventually cooled to 20-30°C in the rheometer water jacket.

As shown in Figure 6, at higher temperatures (50-70°C) the suspension containing 30 wt% HAP and 2 wt% carrageenan had low viscosity. It formed a gel around 40°C, indicated by the sudden increase of viscosity. Below 40°C, the gel was fractured by applied shear force, resulting in the decrease of viscosity. A 2 wt% carrageenan solution without HAP showed a similar temperature dependence (Figure 6). This thermal gelation property made the HAP-carrageenan suspension a good candidate as self-sitting bone filling materials. It was fluid-like and injectable above 45°C, and formed a rigid gel at body temperature of 37°C.

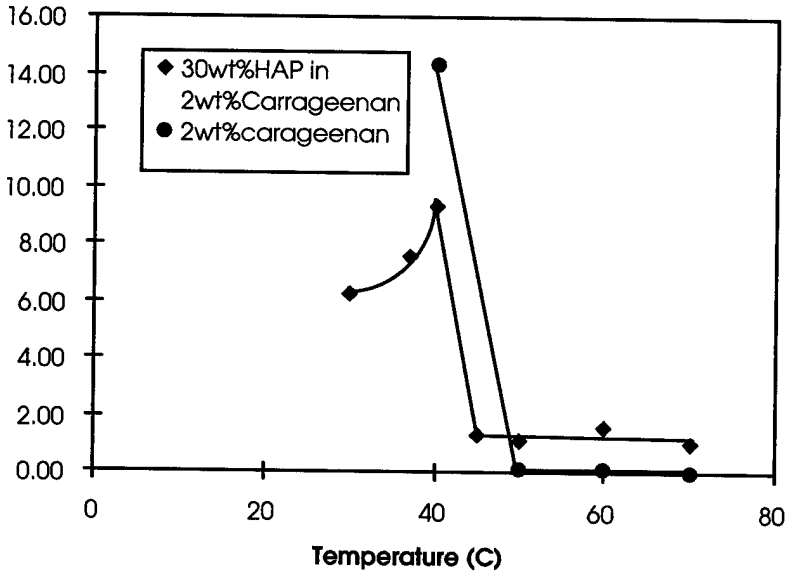

Figure 6. Dependence of Viscosity on Temperature in HAP-Carrageenan Suspensions.

A bioresorbable calcium phosphate phase, β-tricalcium phosphate (TCP) obtained from BassTech International, was tested and compared to HAP. Particle size analysis was performed by light scattering using a Microtrac Full Range Analyzer and by scanning electron microscopy (SEM). The SEM images are shown in Figure 7a and 7b. HAP formed agglomerates which consisted of needle-like primary particles in the size range of 0.2 - 0.5 μm. β-TCP had spherical primary particles between 1 and 2 μm. This difference in morphology had a significant effect in the viscosity and gel strength of the carrageenan paste systems. As shown in Figure 8, an increase of β-TCP solid loadings up to 40 wt% had little effect on the resulting viscosity, in contrast to HAP.

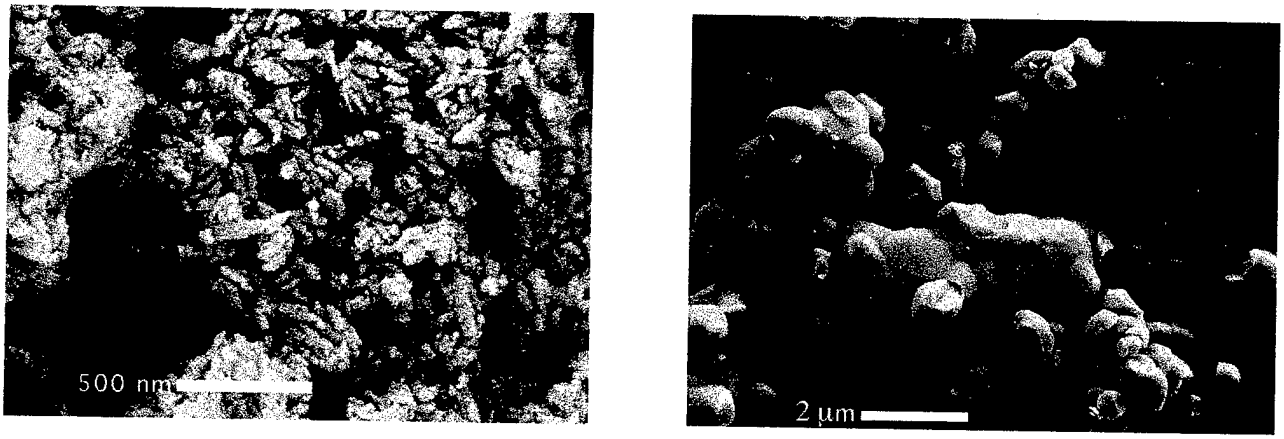

Figure 7. SEM Images of (a) HAP and (b) β-TCP.

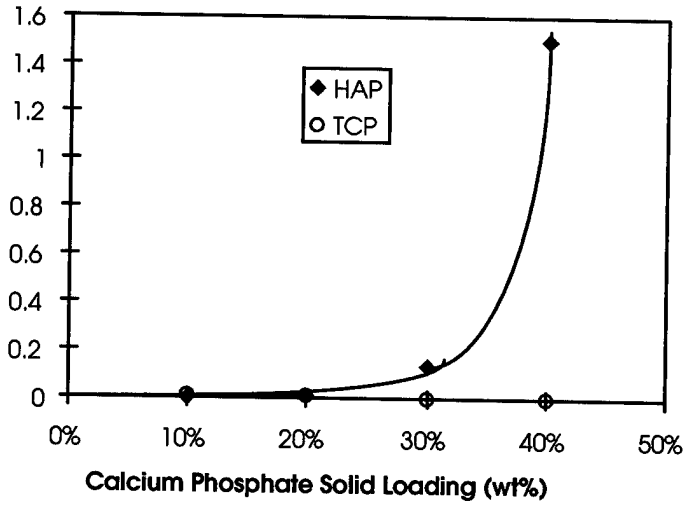

Figure 8. Viscosity of HAP and β-TCP in 0.4 wt% Carrageenan at 25°C, Shear Rate 116 s^{-1}.

Results from the temperature dependence viscosity measurements (Figure 9) indicated that β-TCP-carrageenan paste formed a gel at the same temperature (40°C) as the HAP-carrageenan paste. However, the viscosity of the β-TCP-carrageenan paste was significantly lower than that of the HAP-carrageenan paste. This suggests that the gelation temperature is mainly controlled by the binder phase, while the rheological properties and potentially the mechanical strength is dominated by the morphology of the ceramic phase.

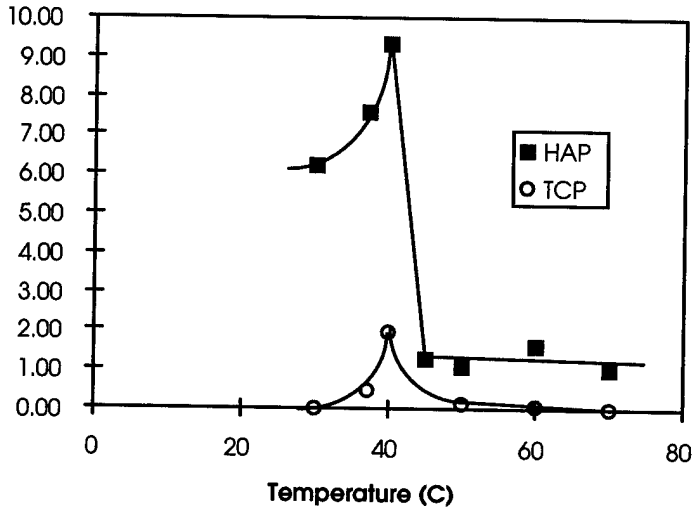

Figure 9. Viscosity of 30 wt% HAP and β-TCP in 2 wt% carrageenan as a function of temperature. T=25°C, shear rate=116s^{-1}.

2.2 Component Fabrication

2.2.1 Materials and Methods

The polysaccharide solutions that were evaluated during this task, xanthan gum, locust bean gum and *k*-carrageenan, were prepared as previously discussed in Section 2.1.1 - Materials and Methods. The xanthan gum-, locust bean gum-, and carrageenan-HAP, zirconia and alumina pastes were also prepared as previously discussed in Section 2.1.1 - Materials and Methods. Once the polysaccharide-HAP pastes were prepared, they were vibrated using a WhipMix Vibrating Table into a white rubber mold (tooth root

or femoral head), immersed into a saline or PBS bath, cross-linked or cured in the bath for five days, and removed for evaluation.

2.2.2 Results and Discussion

Due to the low solubility of *k*- carrageenan at room temperature and the difficulty of handling warm solutions without premature cooling and subsequent gelation, *k*- carrageenan was eliminated as an option for a fabrication binder system. Even after filtration of the endosperm, the locust bean gum solutions were still visibly turbid. If left in the solution for fabrication, residual endosperm would become entrapped. The entrapped endosperm would be removed during subsequently thermal processing, i.e., binder burnout, but would leave behind unwanted porosity and ash. Thus, locust bean gum was also eliminated as a fabrication binder option.

Xanthan gum showed promise as a HAP fabrication binder system, but HAP solid loadings were limited to 43 wt% in order to maintain good flow into the intricacies of the mold. In addition, xanthan gum chelated in the presence of phosphates further limiting solid loadings. Since successful fabrication relies on high solids content to help maintain as-formed strength and decrease binder content for successful densification, xanthan gum was also eliminated as a HAP fabrication binder system. Xanthan gum proved to be a much more effective binder for the zirconia and alumina systems. Several shapes, including a femoral head and a disk, were demonstrated. However, due to limited resources, the component fabrication task was discontinued.

2.3 Characterization of Materials Properties

2.3.1 Materials and Methods

HAP-polysaccharide paste samples prepared for mechanical testing, microstructure evaluation, and porosity studies were prepared as previously discussed in Section 2.1.1 and crosslinked in a simulated blood plasma electrolyte solution overnight at room temperature. The simulated blood plasma electrolyte solution contained 3 milli-molar (mM) KCl, 1.5 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 138 mM NaCl and 2.5 mM $CaCl_2$. It was adjusted to pH 7.4 by addition of NaOH solution before use. Compressive modulus measurements were performed using a Perkin-Elmer Dynamic Mechanical Analyzer. The microstructure and porosity samples were subsequently cut into small rectangular samples

with dimensions approximately 2.5 cm x 0.5 cm x 0.3 cm. The samples were frozen in liquid nitrogen and then dried in a vacuum (1 x 10^{-3} mm of mercury) at -12°C for at least 24 hours.

2.3.2 **Results and Discussion**

2.3.2.1 Compressive Modulus

Compressive modulus is a measure of the resistance of the sample to compression and is an indicator of stiffness or rigidity. Stress and strain were determined for three HAP-carrageenan samples containing 2 wt% carrageenan and various amount of HAP. Stress was defined as F/A where F was the force applied on a cylindrical disc sample, and A was the sample surface area. Strain was defined as $\Delta H/H_o$ where H_o was the initial height of the sample, and ΔH was the height difference before and after force F was applied. The results are shown in Figure 10. Compressive modulus can be estimated from the slope of each plot. The sample containing 2 wt% carrageenan with 0.0 wt% HAP had the lowest compressive modulus, which was estimated to be 7.79×10^3 Pa. The addition of up to 30 to 40 wt% HAP increased the compressive modulus to $1.13\text{-}1.17 \times 10^4$ Pa. The increase in compressive modulus with increasing ceramic content may be explained by data reported in the polymers and plastics literature. As reinforcements are added to a polymeric material, increases in impact and tensile strengths are seen as well as tensile modulus (7 - 8). This trend is more pronounced when the reinforcement has a high aspect ratio, such as a fiber or whisker. This means that the material becomes stiffer. An increase in compressive modulus or an increase in stiffness was what was shown here with increasing HAP content.

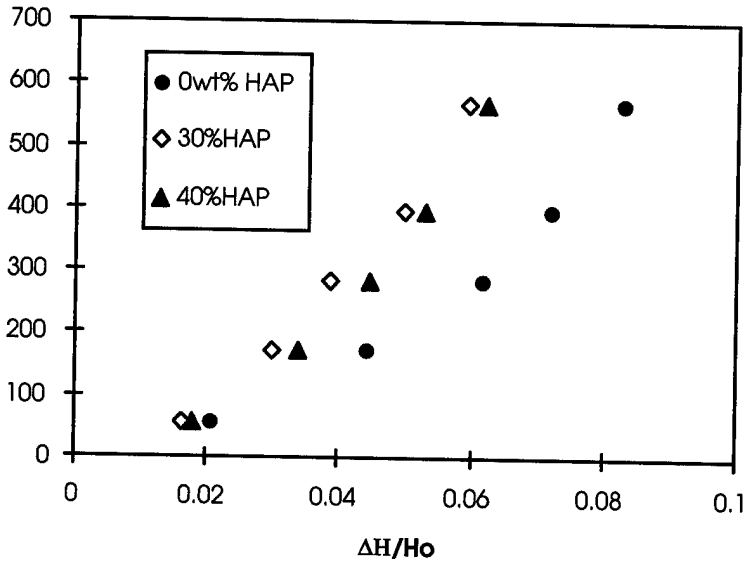

Figure 10. Measured Stress (F/A) vs. Strain ($\Delta H/H_o$) of HAP-Carrageenan Composites.

2.3.2.2 Microstructure and Porosity

The microstructure and porosity of HAP-carrageenan pastes were studied by scanning electron microscopy (SEM). To preserve the as-gelled structures, the paste samples were cut, frozen in liquid nitrogen, then dried in a vacuum at -12°C for at least 24 hours. The dimension of each piece was measured using a caliper before and after freeze drying to determine shrinkage. While the samples containing 30 wt% or more HAP showed less than 5% linear shrinkage, a sample containing 10 wt% HAP shrank 10-20%, indicating that the lower solid loaded structure collapsed during freeze drying. The SEM images of 30 wt% and 40 wt% HAP in 2 wt% carrageenan are shown in Figure 11. The structure of the 30 wt% HAP sample (Figure 13 (a)) contained HAP agglomerates of 5-10 ?m and pores ranging from 5-15 μm. Increasing the HAP solids loading to 40 wt% (Figure 13 (b)) produced a denser material and reduced the pore size to less than 5 ?m.

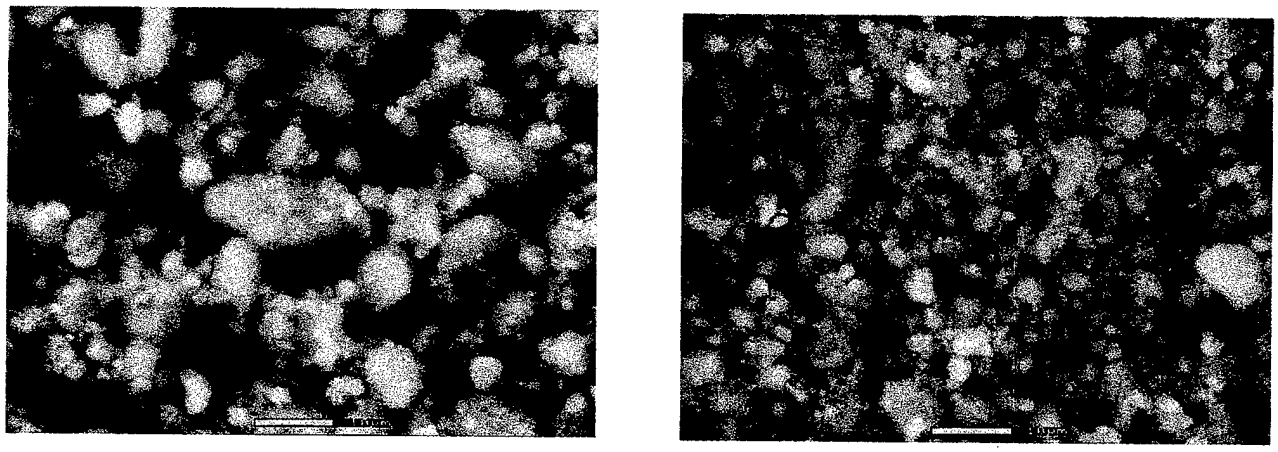

Figure 11. Microstructures of (a) 30 wt% HAP in 2wt% Carrageenan and (b) 40 wt% HAP in 2 wt% Carrageenan.

2.4 Biological Response

2.4.1 Materials and Methods

HAP-polysaccharide paste samples prepared for dissolution experiments were crosslinked in simulated blood plasma electrolyte solution for overnight at room temperature. The simulated blood plasma electrolyte solution contained 3 mM KCl, 1.5 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 138 mM NaCl and 2.5 mM $CaCl_2$. It was adjusted to pH 7.4 by the addition of NaOH solution before use.

HAP dissolution kinetics was measured using constant composition (CC) method at 25°C (9). The in-vitro carrageenan leaching experiments were conducted in the simulated blood plasma electrolyte solutions at 37°C. The concentration of leached carrageenan was determined by a colormetric method using methylene blue (10).

2.4.2 Results and Discussion

2.4.2.1 HAP Dissolution by Constant Composition Method

The kinetics of HAP dissolution of HAP-carrageenan pastes was studied by the CC technique. An undersaturated solution containing 0.125 mM $CaCl_2$, 0.075 mM KH_2PO_4 and 0.15 mM NaCl was prepared by mixing stock solutions of 0.1M $CaCl_2$ and 0.1M KH_2PO_4 in 0.15M NaCl. The solution pH was adjusted to 6.5 using 0.1M KOH solution. Nitrogen saturated with water vapor was purged through the prepared solution to exclude carbon dioxide. A combination pH electrode (Corning) was used as a

probe to monitor solution pH. A 0.083g sample containing 30 wt% HAP and 2 wt% carrageenan was added to the undersaturated solution to initiate the dissolution experiment. The solution pH was kept constant by the potentiostatic controlled addition of 0.30M NaCl and 0.007M HCl. The rate of dissolution was calculated from the rate of addition of the acid after correcting for the volumes required to maintain constant pH. For comparison, the dissolution rate of 0.025 gm HAP powder, which is equivalent to the amount of HAP in the HAP-carrageenan paste sample was measured using the same technique (Figure 12). The CC results indicate both samples have high initial dissolution rates which slowed down as more and more calcium and phosphate ions accumulated in the solutions. HAP-carrageenan paste (3.4 mol Ca/m^2-min) had a lower initial dissolution rate than the free HAP powders (5.7 mol Ca/ m^2-min). This was probably due to the fact that carrageenan bonds HAP particles together thus reducing the effective surface area for dissolution to occur. As the carrageenan gradually leached out, the HAP-carrageenan paste became less bound and acted more like free particles.

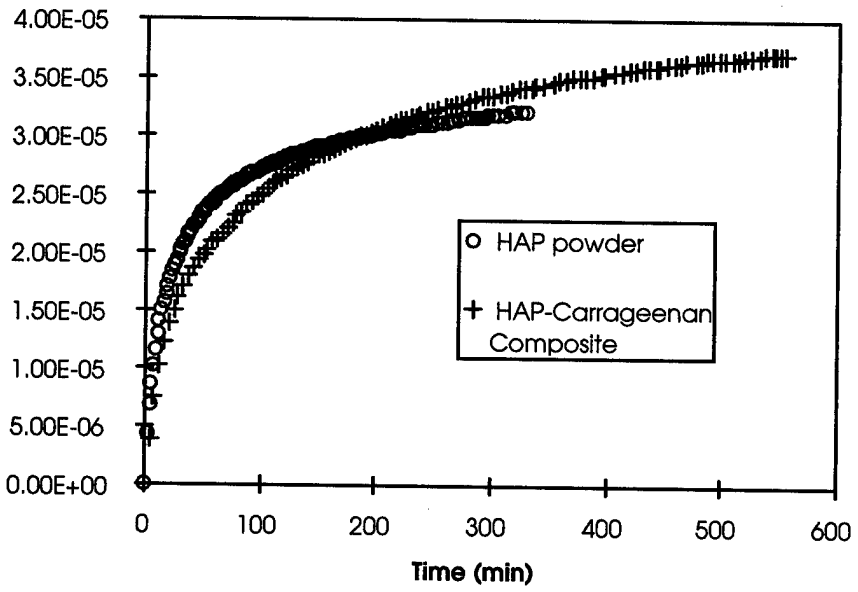

Figure 12. Dissolution of HAP-Carrageenan Paste and HAP Powder at pH 6.5, [$CaCl_2$]=0.125 mM, [KH_2PO_4]=0.075 mM, [NaCl]=0.15 mM.

2.4.2.2 Carrageenan Leaching Studies in Simulated Blood Plasma Electrolyte Solutions

Suspensions containing 2 wt% carrageenan and 0 to 40 wt% HAP were prepared at 75°C. They were poured into small weighing dishes and formed blocks upon cooling to 37°C. After weighing each block (approximately 8 grams), they were placed in 20 ml of simulated blood plasma solution at 37°C. The solution was collected and replaced with new solution every 20 - 24 hours. The HAP powder that remained in the solution was separated out via centrifugation. The supernatant was subsequently diluted and mixed with 0.41 mM methylene blue solution. The carrageenan concentration was determined by measuring UV adsorption at 559 nm against a standard calibration curve. The result is shown in Figure 13.

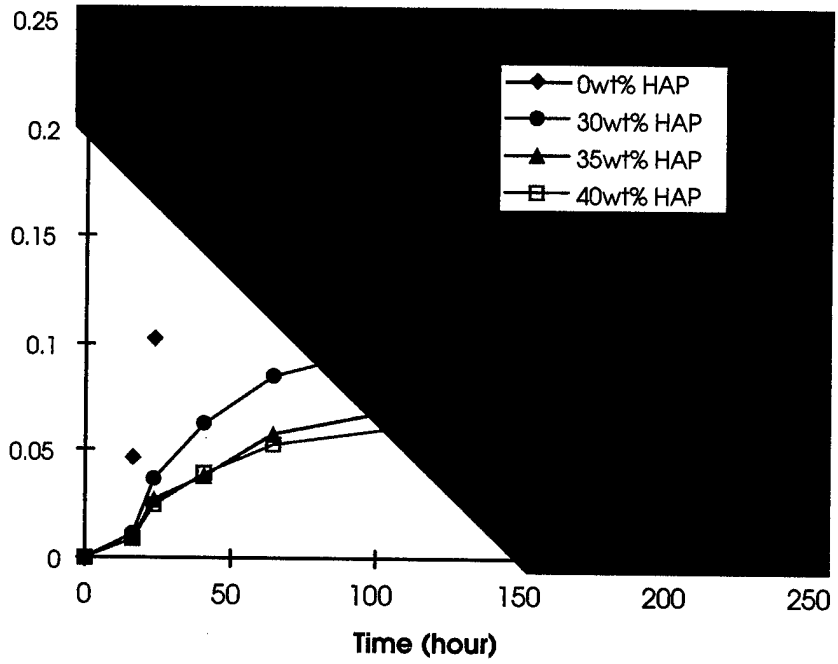

Figure 13. Fraction of Carrageenan Leached as a Function of Time.

Among the paste samples tested, the sample containing 0.0 wt% HAP was softened and broken down after 2 days. Therefore, no data was available after this period. At 30 wt% and higher HAP loadings, the blocks maintained their shape. The results indicated that as the HAP solid loadings increased, the carrageenan leach rate slowed. The negatively charged *k*-carrageenan is known to be gelled by potassium ions and slightly by calcium ions. One possible mechanism is that potassium saturated and calcium rich

HAP surfaces provided binding sites for carrageenan. This interaction reduced the carrageenan leach rate. As the HAP solid loadings increased, more sites were available for binding. An alternative mechanism is that the calcium ions dissolved from the HAP to bind to the carrageenan subsequently controlled the leach rate. Our current results seem to suggest the first mechanism. Further investigation is under way to distinguish between the two mechanisms.

2.4.2.3 *In-vivo* Evaluation of Bone Fillers

A sixth task was received from the MRMC office to evaluate the ability of the bone pastes that were evaluated on Tasks 1 - 5 to facilitate new bone formation in-vivo. A sub-contract was established with University of Toronto - Centre For Biomaterials to evaluate chitosan and carrageenan-calcium phosphate pastes and scaffolds in a rat bone model. Carrageenan and Chitosan containing composite pastes, with powdered hydroxyapatite, were used for the *in vivo* analysis. Chitosan was supplied as a 2% viscous solution that turned water-like after sterilization. This change implied a change in chemistry. The freshly prepared sterile Carrageenan paste was much thicker and did not become liquid-like when heated to 75°C. Instead; it remained paste-like regardless of temperature. Sterile Carrageenan pastes were dispensed into 1cc syringes and heated to 75° C prior to placement in surgical site. Pellets were surgically introduced as monoliths.

Samples were implanted bilaterally into the mid-diaphysis of Wistar rats (approximately 125grms) according to protocols approved by the Animal Care Committee of the University of Toronto. The femora were prepared with a single drill hole (No. 4 round bur) to accept the sample. The wounds were closed in multiple layers and the skin stapled. Following either one or three weeks, the animals were killed and osteotomies were performed on the femora to remove the operative site and approximately 3mm of surrounding bone. The tissues were fixed, decalcified (formic acid), and dehydrated using standard methods to prepare for Hematoxylin and Eosin light micrography.

Both Carrageenan and Chitosan preparations demonstrated a florid inflammatory reaction at 1 week, which diminished but were still evident at 3 weeks. At 3 weeks, the residual inflammation was greater in the Carrageenan-containing samples than the Chitosan-containing samples. All samples showed evidence of new bone growth on the surface of the hydroxyapatite particulate. This was more evident at 3 weeks than at 1 week. In some cases, with Carrageenan-containing samples this bone response was so marked

that the marrow cavity was almost obliterated by new bone formation. While there was considerable evidence for osteoclast resorption of the surrounding bone, and at 3 weeks, osteoclasts present on the hydroxyapatite (HA) particulate, there was no evidence of the osteoclasis of the HA particulate. Fibrous reaction tissue was also evident, but more pronounced in the Chitosan-containing samples.

Neither the Carrageenan nor Chitosan containing composite pastes would appear to be suitable candidate bone-substitute materials. Hydroxyapatite is known to be biocompatible and elicits a minimal inflammatory response. It is reasonable to assume, therefore, that the florid inflammatory responses seen with these pastes were due to the Carrageenan or Chitosan respectively.

2.5 Development Of Resorbable Tricalcium Phosphate/Chitosan Composites For Bone Tissue Replacement

The purpose of Task 5 was to investigate stimuli-sensitive, reversible gels as polymeric components of polymer/HAP(TCP) composites. A characteristic feature of the reversible gels is that they exhibit a phase transition between liquid and solid state upon a change in temperature or pH. As a consequence of this transition, a reversible polymer/HAP(TCP) composite would be in the form of an injectable paste at room temperature and would harden to make a solid material at body temperature and/or pH. This would create a possibility of a custom-tailored implant for any shape of the bone defect. Placed, for example, in the fracture side, these materials would provide a scaffolding into which the new bone would grow. Other polymer/ceramic composites rely on pre-formed shapes that may not satisfy the requirements of particular cases.

As a specific example of pH-reversible polymeric gels, chitosan solutions exhibit a viscous liquid-gel transition around pH 7. Chitosans are a family of biodegradable and biocompatible cationic polysaccharides produced commercially by the partial deacetylation of chitin obtained from the reprocessing of seafood waste. Chitin is the second most plentiful natural biopolymer, next to cellulose (11). Chitosans are a (1,4)-linked 2-amino-2-deoxy-B-D-glucans and can be prepared by N-deacetylation of chitin. Members of chitosan family differ in terms of their molecular weight and degree of deacetylation. In recent years, chitosans were investigated for many diverse medical applications such as wound dressings, contact lenses and materials for cell encapsulation (12 - 13). It was also shown that treatment of various canine tissues with chitosan solution resulted in the inhibition of fibroplasia and enhanced tissue regeneration (14).

Application of a composite containing chitosan and hydroxyapatite granules as a bone filling paste was recently described by Maruyama and Ito (15 - 16). The self-hardening paste was prepared by using a combination of chitosan, hydroxyapatite granules, ZnO and CaO. Loading of 55 wt% of HAP granules was necessary to obtain compressive strengths comparable with cancellous bone from tibial eminentia. The setting kinetics and the physical form, however, makes these pastes not suitable for injectable formulations.

In this task, chitosans were investigated as new biodegradable component of polymer-ceramic composites suitable for injectable, resorbable templates for bone tissue regeneration. The rationale of using chitosans for this purpose is based on the fact that chitosan solutions gel in response to pH change from slightly acidic to physiological.

The unique aspect of this novel system is that at pH lower than 6.5 the chitosan-ceramic suspension is a paste-like moldable system and at physiological pH the polymer undergoes a phase transition resulting in entrapment of ceramic component within the reversible gel matrix. Thus, application of pH-reversible gels would enable the creation of an implant which may be tailored to any shape of a bone defect needed to be filled.

2.5.1 **Materials and Methods**

High molecular weight (HMW) chitosan, 1.1-1.6x10^6 daltons (D), was purchased from Aldrich Chemical Company, Inc., low molecular weight (LMW) chitosan, 70 KD, was purchased from Fluka Chemie. Chitosans were dissolved in 0.1N HCl and purified before use by precipitation into acetone/water mixture. Calcium phosphate (HAP) was purchased from Aldrich Chemical Company, Inc., and tricalcium phosphate (TCP) was obtained from BassTech International. Bovine serum albumin (BSA), 5-fluorouracyl (5-FU), and lysozyme (hydrolytic enzyme) were purchased from Sigma Chemical Company and used in the as-received state. Phosphate buffered saline (PBS), pH 7.4 was used to prepare all polymer-ceramic suspensions and as a release medium in BSA and 5-FU release experiments.

2.5.1.1 Preparation of Chitosan-HAP and Chitosan-TCP Suspensions

Solutions containing 0.5-2 wt% of purified chitosan were prepared in 0.1N acetic acid. Chitosan-HAP or -TCP suspensions were prepared by mixing the ceramic component in the chitosan solution on a magnetic stir plate. Suspensions containing 20-45 wt% of HAP and 30-50 wt% of TCP were prepared.

2.5.1.2 Rheological Properties

Rheological properties of chitosan-calcium phosphate suspensions were investigated using a Bohlin VOR Rheometer. Viscosities of chitosan-HAP suspensions containing 12, 20, 30, and 40 wt% HAP solids were measured at a 116 s^{-1} shear rate.

2.5.1.3 Water Content in Cured Composites

Acidic chitosan-calcium phosphate suspensions (pastes) were cured in PBS at pH 7.4. Disk samples of approximately 15 mm x 4 mm thick were cut out with a corkborer. Water content in the initial cured samples and rehydrated samples was determined gravimetrically. For the initial cured samples the following equation was used to calculate $W_{\%}$,percent of water in the sample:

$$W_{\%} = (W_i - W_d)/W_i \times 100\%,$$

where W_i denotes the initial weight of the cured paste sample and W_d denotes the weight of that sample in a dried state. For the rehydrated sample the following equation was applied:

$$W_{\%R} = (W_R - W_d)/W_R \times 100\%,$$

where $W_{\%R}$ = denotes a percent of water in a wet sample that was rehydrated from a dried state. Rehydration of freeze-dried and room-temperature dried samples was compared.

2.5.1.4 Microstructure

Microstructure of chitosan-calcium phosphate composites was investigated by field emission scanning electron microscopy (FE SEM) with LEO 982. The effect of ceramic phase content on the porosity and pore size was investigated. The microstructure of freeze-dried and room-temperature dried samples was also compared to evaluate the effect of drying technique on the pore size of the composites. Samples

were freeze-dried by immersion in liquid nitrogen and removal of water through vacuum drying at approximately -12°C for 26 hours. Room temperature dried samples were prepared by placing samples at room temperature and ambient atmosphere until measured weight loss was constant.

2.5.1.5 Compressive Modulus

Experiments were performed using the Perkin-Elmer Dynamic Mechanical Analyzer (DMA 7e) equipped with a stainless-steel parallel plate measuring system. Compressive modulus was determined by using a uniaxial, unidirectional compressive deformation. The samples, in the form of cylindrical disks, were held in place initially with a minimal static stress, then the static stress was increased. The response of the sample (static strain) was used to calculate the static compressive modulus.

2.5.1.6 Enzymatic Degradation

Enzymatic degradation of chitosan was investigated using lysozyme as the hydrolytic enzyme and crosslinked chitosan gels as model substrates. Crosslinked chitosan gels with very low crosslinking density were obtained by reaction of a 2% chitosan solution in 0.1 M HCl with glutaric aldehyde. The excess of unreacted aldehyde was removed by thorough extraction of resulting gels in water. Small samples of purified gels weighing approximately 0.5 - 0.6 grams were placed in PBS solution containing 1 mg/ml of lysozyme. The progress of gel degradation was evaluated based on the sample weight change with time. Percent degradation at time (t) was calculated as follows:

$$\% \text{ degradation} = (W_o - W_t)/W_o \times 100\%$$

where W_o denotes the initial weight of the sample and W_t denotes the weight of the sample at time t .

2.5.1.7 Release of Model Compounds

The release of 5-FU and BSA from chitosan-HAP or -TCP composites was conducted in PBS, pH 7.4 at 37°C. Cylinder shaped samples of the composites were cured in a small diameter dialysis tubing. The amount of released compounds was determined by monitoring UV absorption of the release medium at 266 and 280 nm for the 5-FU and BSA, respectively. To maintain sink conditions, samples were transferred into fresh release medium at predetermined time intervals. The release kinetics data was reported as fraction of the total amount released by weight versus time.

2.5.2 **Results and Discussion**

In order to obtain chitosan-calcium phosphate composites suitable for the injectable, resorbable scaffolds for bone tissue regeneration, several properties of the composites need to be investigated and optimized. The most important requirements are the following: biocompatibility of the materials and their degradation products, injectability of the polymer-ceramic suspensions, suitable gelation kinetics of the composites, controlled degradation rates, ability to release small and large therapeutic agents and finally osteoconductivity. As a consequence of the above requirements, we have concentrated on investigation and optimization of the following properties: rheological properties of the suspensions, and water content, porosity, compressive strength, and degradation rate of the cured composites. The ability of the composites to release therapeutic agents was also evaluated.

2.5.2.1 Chitosan-Calcium Phosphate Suspensions: Preparation and Rheological Properties

Chitosan dissolves in diluted acids at pH 2-3 but the pH of the obtained solution may be titrated up to a pH of 6 with no change in polymer solubility. Therefore, chitosan-ceramic suspensions may be prepared at a wider pH range, i.e. 2-6. The pH of chitosan solution is important since it affects the solubility of the calcium phosphate phase of the composite.

Rheological properties of chitosan solutions and chitosan-calcium phosphate suspensions were investigated as a function of chitosan concentration, molecular weight, content and type of a calcium phosphate phase (HAP or TCP).

As illustrated in Figure 14, HMW chitosan-HAP suspensions containing up to 40 wt% of HAP phase exhibited viscosities and flow suitable for injectable bone paste applications.

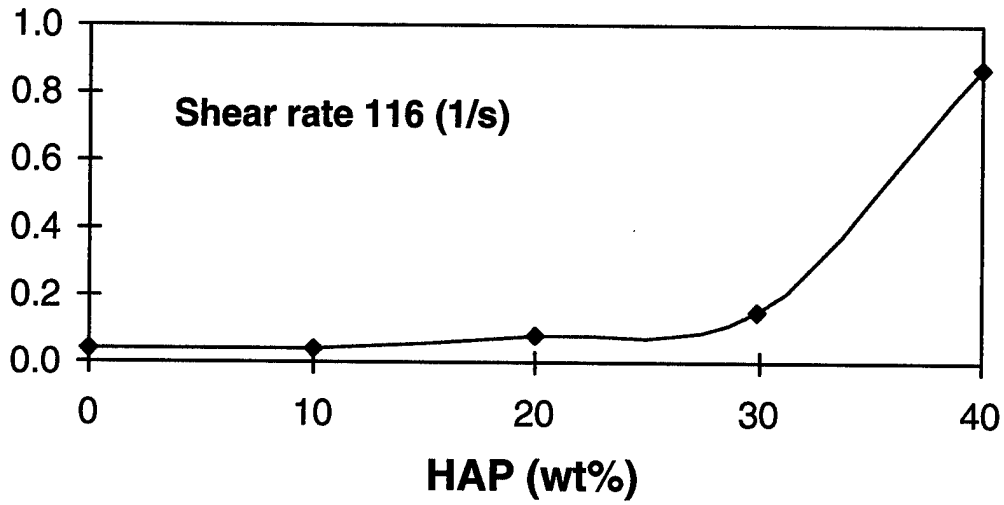

Figure 14. Viscosity of Chitosan-HAP Suspensions as a Function of HAP Solids Loadings

2.5.2.2 Water Content in Cured Composites

When exposed to physiological pH, the paste-like chitosan-calcium phosphate suspensions change into solid polymer-ceramic composites with the ceramic components entrapped effectively within the reversible gel matrix. The chitosan gelling transition takes place around pH 7, hence, chitosan-calcium phosphate suspensions may be cured at physiological pH. In this studies, chitosan-calcium phosphate suspensions were cured in PBS solutions at pH 7.4. The water content in the initial and rehydrated samples was determined. Results for the rehydrated samples are important in the case of the preformed composites only.

So far, the water content was determined for the chitosan-HAP composites containing HMW chitosan with 30, 40 and 45 wt% of HAP. Results are presented in Figures 15 and 16. As shown in Figure 15, each composite demonstrated lower water content in a rehydrated state. This behavior may be related to the additional entanglements of polymer chains created upon drying. Such entanglements would prevent the dry composite scaffold from "swelling" to its original volume in a wet state. These results suggest that preformed scaffolds may demonstrate slightly lower water content after re-swelling in vivo. In Figure 18, the effect of ionic strength on the water content in the initial cured composites is illustrated by a comparison between samples cured in water and in PBS. Overall, chitosan-HAP composites

demonstrated high water content in a cured state. High water content and porosity of the cured composites are necessary to promote cell anchoring and proliferation.

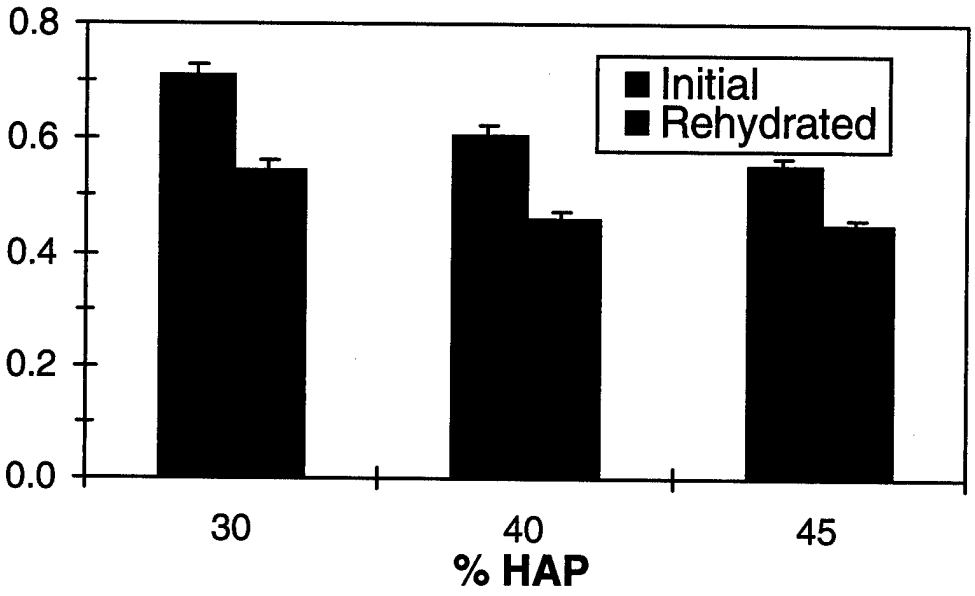

Figure 15: Swelling of Chitosan-HAP Composites in Phosphate Buffered Saline (PBS): Initial and Rehydrated Samples.

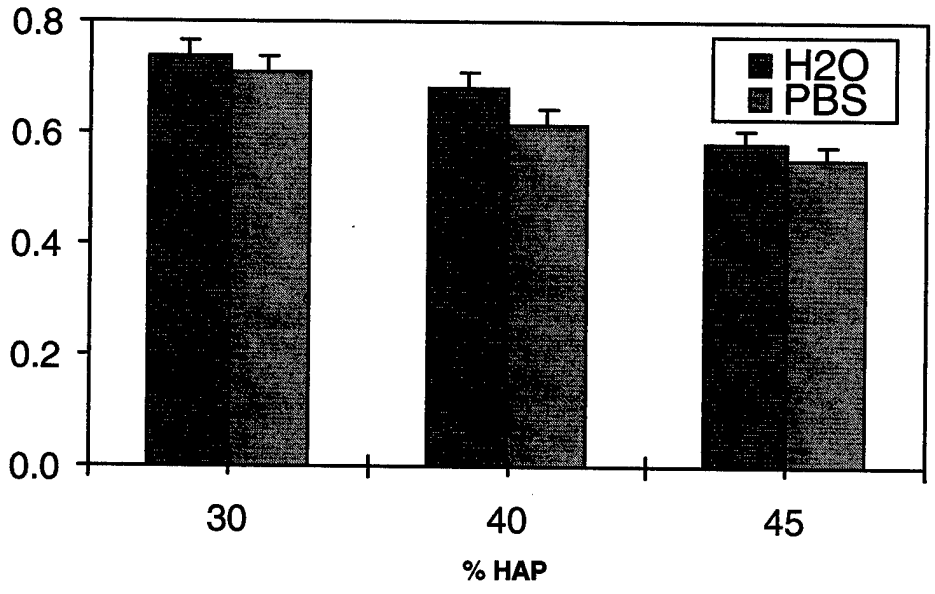

Figure 16: Water Content in Chitosan-HAP composites: Effect of Ionic Strength.

2.5.2.3 Microstructure of Chitosan-HAP Composites

The microstructure of chitosan-HAP composites was investigated by field emission SEM. The effect of ceramic phase content on the porosity and pore size was investigated in a freeze-dried composites. Figure 17 illustrates a relatively dense structure of the freeze-dried chitosan-HAP composite containing 40 wt% of the ceramic phase. In contrast, the composite containing only 20 wt % of HAP shows much higher porosity as well as the pore size as shown in Figure 18. Higher magnification of this composite (Chitosan-HAP 20) shown in Figure 19 clearly illustrates the composite's porous structure with polymer strands binding together the small particles of the ceramic phase.

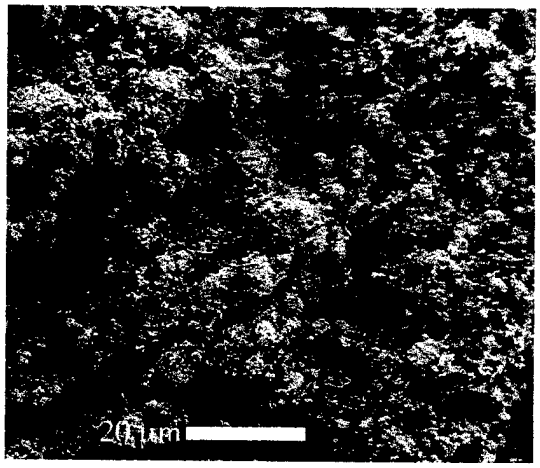

Figure 17. Freeze-Dried Chitosan-HAP 40 Composite.

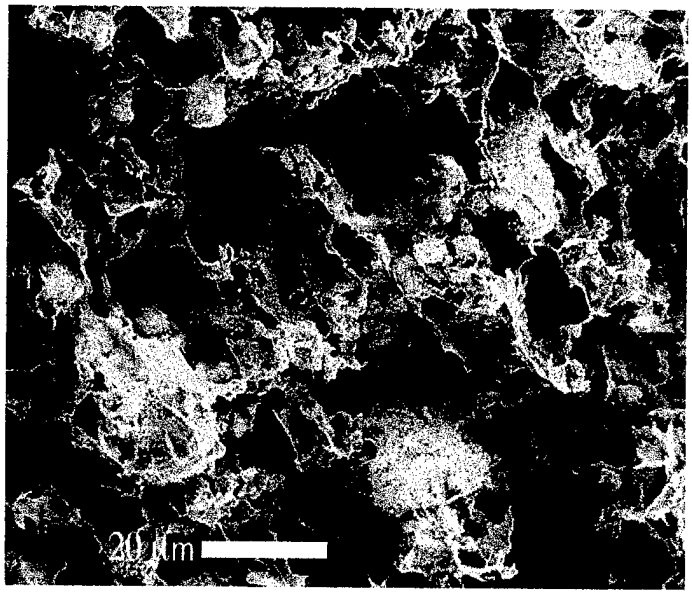

Figure 18. Freeze-Dried Chitosan-HAP 20 Composite.

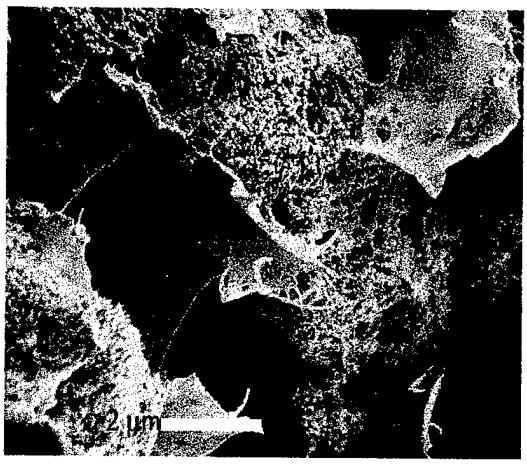

Figure 19. Freeze-Dried Chitosan-HAP 20 Composite at Higher Magnification.

To evaluate the effect of drying method on the pore size of the composites, the microstructure of freeze-dried and room-temperature dried samples was compared. The results are illustrated in Figure 20 showing the structure of a room temperature dried chitosan-HAP 40 composite, and 21 Figure 21 showing the structure of the same composite but as a freeze-dried one. As it is clearly visible, freeze-drying of the composite resulted in a more porous structure. Immersing in liquid nitrogen, freezes the sample structure and polymer chains in their expanded position, as they are in a swollen sample. If the water is removed by freeze drying, so the sample is kept in a "frozen" state, the polymer chains can not relax and collapse. However, at room temperature, polymer relaxation is possible, and the structure collapses slowly upon drying.

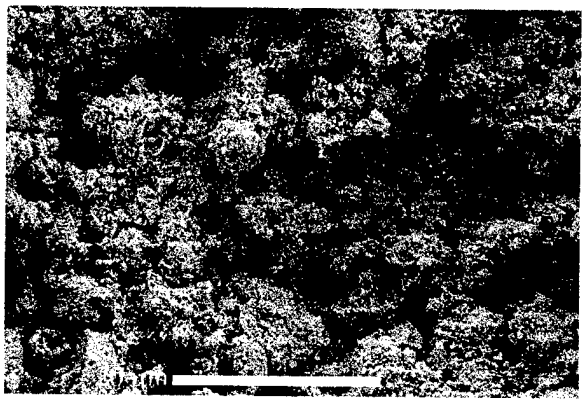

Figure 20. Room Temperature Dried Chitosan-HAP 40 Composite.

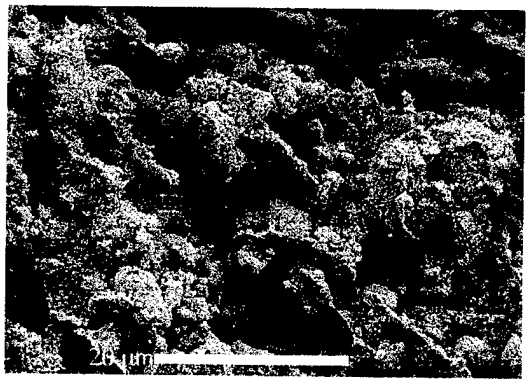

Figure 21. Freeze Dried Chitosan-HAP 40 Composite.

2.5.2.4 Compressive Modulus

Compressive modulus is a measure of the resistance of the sample to compression and is an indicator of stiffness or rigidity. Relative compression moduli were determined to assess the changes in mechanical properties of the polymer-ceramic composites resulting form the gelation of the polymeric component. The compressive moduli were determined for a series of polymer-ceramic composites differing in composition in terms of ceramic (HAP or TCP) as well as polymeric components (LMW or HMW chitosan). Results are presented in Figures 22 - 24. The effect of solids loading, i.e., the amount of ceramic phase in the composite, on the compressive modulus of HMW chitosan-HAP composite is illustrated in Figure 22. Composites with higher solids loading, 40 wt% , demonstrated higher relative compressive modulus as demonstrated by a steeper slope of the stress strain curve. A similar trend was observed for the chitosan TCP composites. The effect of the ceramic phase type is illustrated in Figures 23 and 24. Composites containing HAP demonstrated higher relative compressive moduli than composites containing TCP. The trend was observed for both chitosans, HMW (Figure 25) and LMW (Figure 24). As shown in earlier microstructural examination of HAP and TCP (Figure 7), the HAP powder had a whisker morphology versus the spherical morphology of the TCP starting powder. It is unknown exactly why this trend occurs at this time, however, it could be surmised that the HAP whisker morphology provided a "stiffening" of the resulting structure as discussed in Section 2.1.2.

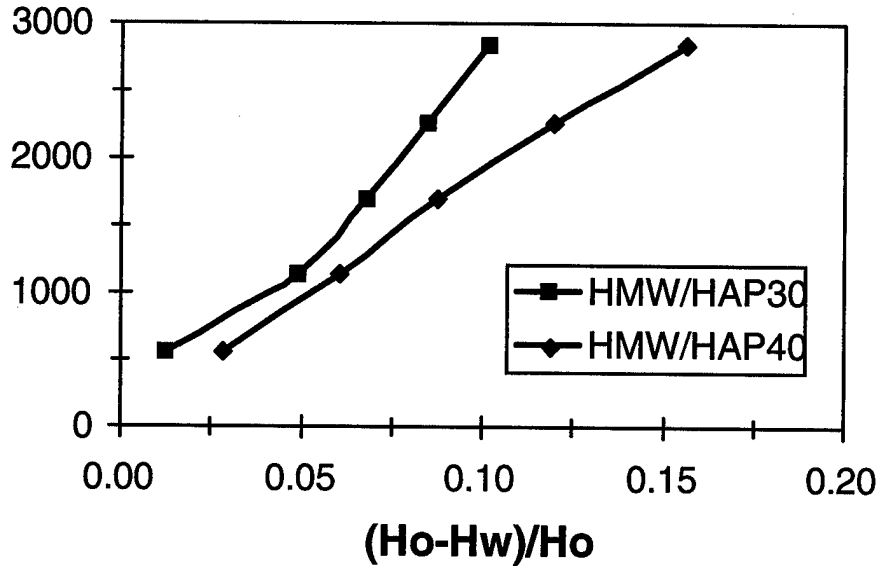

Figure 22. Compression Modulus of Chitosan/HAP Composites: Effect of Solids Loadings.

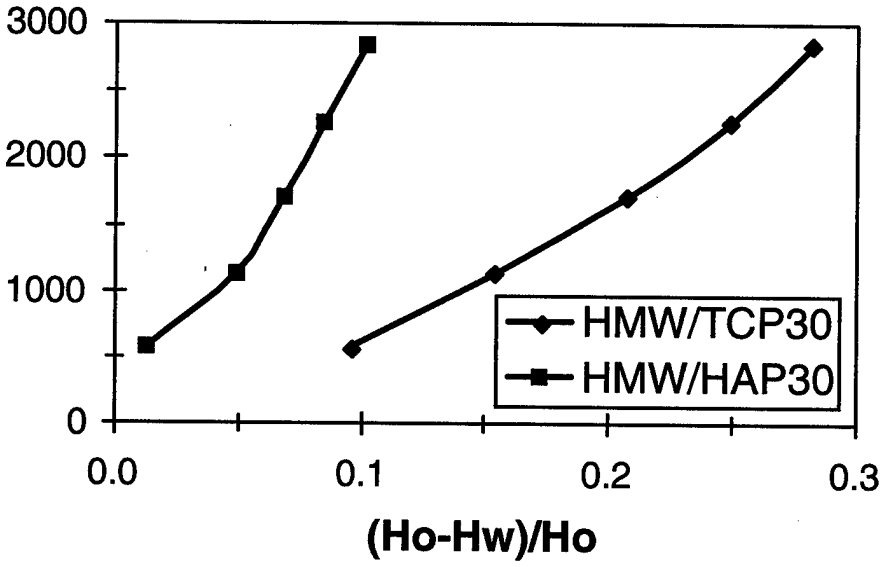

Figure 23. Compression Modulus of HMW Chitosan-CaP Composites: Effect of CaP Phase.

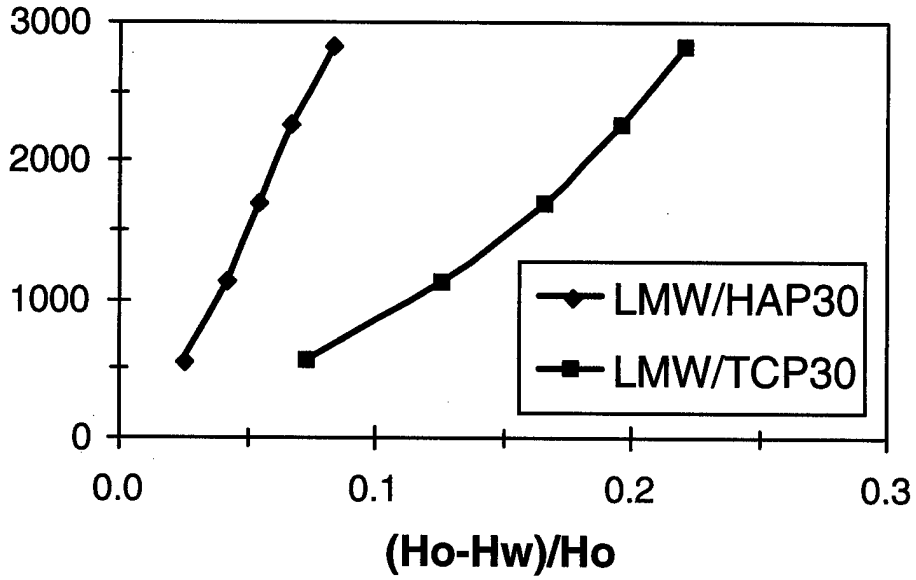

Figure 24. Compression Modulus of LMW Chitosan-CaP Composites: Effect of CaP Phase.

2.5.2.5 Enzymatic Degradation

As described by S.H. Pangburn et al., soluble chitosan, it's films and crosslinked gels may be degraded by enzymatic hydrolysis catalyzed by lysozyme (17). Lysozyme, a well characterized endo-carbohydraze, hydrolyzes (1-4) glycosidic linkages of chitin and certain bacterial-wall peptidoglycans. It has also been shown, that only chitin and partially deacetylated chitin are good substrates for lysozyme, while completely deacetylated chitin is not degradable (18). One may expect that degree of deacetylation may be used as a parameter controlling the degradation rate of chitosan-ceramic composites.

We have investigated enzymatic degradation of crosslinked chitosan gels obtained by crosslinking with glutaric aldehyde. Crosslinked gels were used as a model matrix for the preliminary degradation studies. Degradation experiments are in progress. Preliminary results demonstrated that about 20% of gel degraded within the first three weeks in solutions containing 1 mg/ml of lysozyme. Degradation process is slow due to the high deacetylation degree of chitosan used for the gel preparation. The gels investigated in our experiments were obtained using chitosan with 85% deacetylation degree.

2.5.2.6 Release of Model Compounds

Different therapeutic and osteoconductive agents may be incorporated into the thermally reversible polymer-ceramic composites as needed. Examples of therapeutic agents include antibiotics for the local treatment of possible infections, anticancer agents for the site specific treatment of bone tumors. Examples of osteoconductive agents include growth factors and bone morphogenic proteins. In order to asses the possibility of using our novel polymer-ceramic composites as delivery vehicles for therapeutic and/or osteoconductive agents we have investigated the release of model low and high molecular weight compounds.

An anticancer agent, 5-fluorouracyl (5-FU) was used as a model low molecular weight compound and bovine serum albumin (BSA) was used as a model macromolecule. The results of the release experiments are presented in Figures 25 and 26. As shown in Figure 25, the release kinetics of 5-FU form chitosan-calcium phosphate composites was not affected by the ceramic phase, i.e., HAP vs. TCP. In both cases almost 90 % of drug was released within 10 hours. As illustrated in Figure 26, the release of 5-FU and BSA demonstrated different kinetics. While 5-FU released from the chitosan-TCP composite within 10 hours, BSA release was much less rapid and lasted for 40 hours. These results may be explained by the differences in the effective size of the releasing molecules. BSA, a macromolecule demonstrated slower release kinetics due to the lower effective diffusion coefficient within the polymer-ceramic matrix. Based on the above results it may be concluded that our chitosan-calcium phosphate composites may serve as a matrix to release macromolecular therapeutic agents including macromolecules.

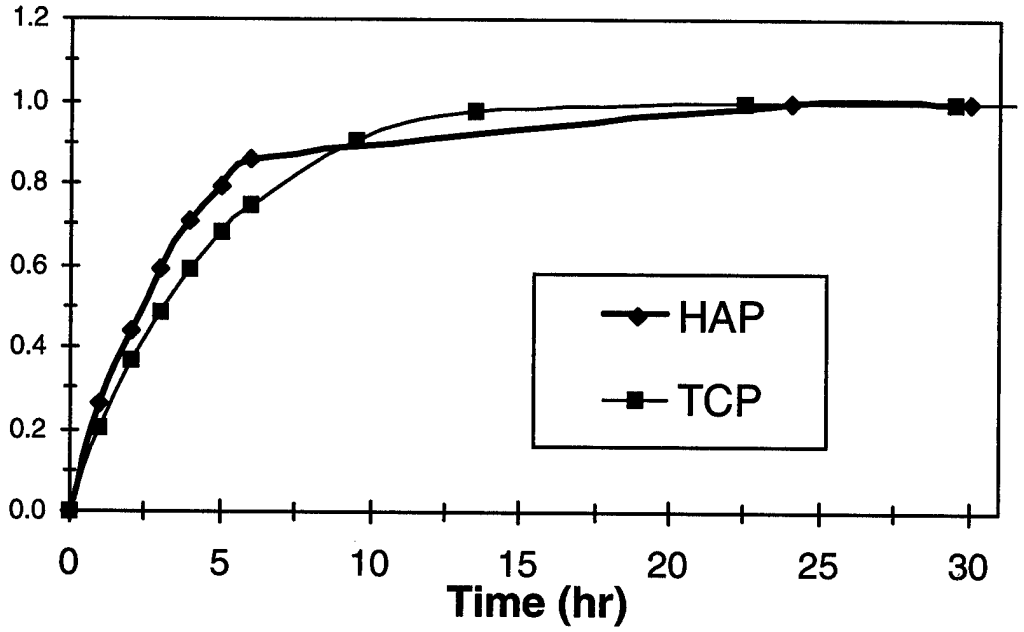

Figure 25. Release of 5FU from Chitosan-CaP Composites.

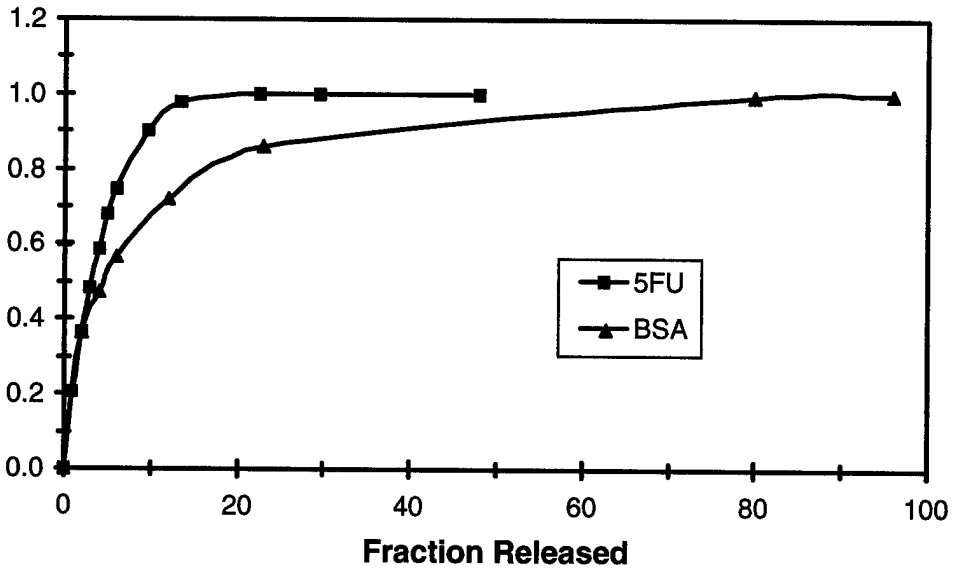

Figure 26. Release of 5FU and BSA from Chitosan-TCP Composites.

3. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated the use of stimuli sensitive polymers as binders in calcium phosphate composites.
- Demonstrated the ability to form composites with different porosities.

- Demonstrated the ability to control rheological properties of the composites.
- Demonstrated the ability to release therapeutic agents from gelled composites.
- Demonstrated the biodegradation of the ceramic/polymer composites.

4. OUTCOMES

- Patent application
- Student research opportunities
- Presentations
- Manuscript
- Interest from several companies on continued research and commercialization.

5. CONCLUSIONS

Our results demonstrated that stimuli sensitive-calcium phosphate composites may be a good candidate for an injectable, resorbable scaffolds for bone tissue regeneration. The rheological properties of the composite suspensions were optimized towards injectability. The water content and porosity of cured composites were aimed to assure cell anchoring and proliferation. Mechanical properties were optimized towards compressive strengths that assure integrity in non-load bearing applications. Preliminary degradation studies in the chitosan-calcium phosphate composites demonstrated slow degradation rates that may be optimized to allow for bone tissue ingrowth before a substantial loss of mechanical strength. Finally, a feasibility of the release of therapeutic agents was demonstrated. Unfortunately, the two polymer materials that were evaluated for in vivo compatibility, showed an inflammatory response to surrounding tissue. Once this subsided, bone formation occurred. However, these may not be suitable polymers and other should be investigated.

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7. APPENDIX

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J Young ((PNNL)

Patent application

Draft Manuscript for J Materials, Materials in Medicine

Attorney Docket
APPLICATION OF Armstrong, Campbell, and Gutowska PATENT

For: POLYMER/CERAMIC COMPOSITES

TECHNICAL FIELD

The present invention relates to a method for constructing an implant by placement of a paste comprising a stimuli sensitive polymer solution carrying a bio compatible ceramic component which hardens under physiological conditions to form a solid implant. The implant may also include a therapeutic agent or a radioisotope.

BACKGROUND OF THE INVENTION

Many researchers have experimented with drug delivery vehicles based on the use of controlled release implant materials. Others have sought to provide improved implants for filling in tissue losses from age or trauma, to hard or soft tissues. Calcium phosphate pastes have been suggested as bone and dental fillers. Gels have been used as control release devices and as fillers. Representative studies are discussed below.

In U.S. Pat. No. 4,188,373, certain polyols are used in aqueous compositions to provide thermally gelling aqueous systems. In these systems the sol-gel transition temperature can be changed by manipulating the concentration of polymer. In U.S. Pat. Nos. 4,474,751; '752; '753; and 4,478,822 drug delivery systems are described which utilize thermosetting gels. In these systems both the gel transition temperature and/or the rigidity of the gel can be modified by adjustment of the pH and/or the ionic strength, as well as by the concentration of the polymer. U.S. Pat. Nos. 4,883,660; 4,767,619; 4,511,563; 4,861,760, and 4,911,926 also disclose gels that deliver pharmaceutical compositions.

In U.S. Pat. No. 4,895,724, compositions are disclosed for the controlled release of pharmacological macromolecular compounds contained in a matrix of chitosan. Chitosan can be cross-linked utilizing aldehydes, epichlorohydrin, benzoquinone, etc. In U.S. Pat. No. 4,795,642, discloses gelatin-encapsulated, controlled-release pharmaceutical compositions, wherein the gelatin encloses a solid matrix formed by the cation-assisted gelation of a liquid filling composition incorporating a vegetable gum together with a pharmaceutically-active compound. The vegetable gums are disclosed as polysaccharide gums such as alginates which can be gelled utilizing a cationic gelling agent such as an alkaline earth metal cation.

Osmotic drug delivery systems are disclosed in U.S. Pat. No. 4,439,196 which utilize a multi-chamber compartment for holding osmotic agents, adjuvants, enzymes, drugs, pro-drugs, pesticides, and the like. These materials are enclosed by semipermeable membranes so as to allow the fluids within the chambers to diffuse into the environment into which the osmotic drug delivery system is in contact. U. S. Pat. No. 5,587,175 teaches a process for forming a protective corneal shield or an ablatable corneal shield or mask in situ comprising administering to the eye of a mammal an aqueous composition capable of being gelled in situ to produce an hyper osmotic, hypo osmotic, or iso osmotic aqueous gel having a controlled pH, said aqueous composition, including at least one film forming polymer; and gelling said film forming polymer in situ to form said protective corneal shield or ablatable corneal shield or mask.

U.S. Pat. No. 3,949,073 discloses injectable atelocollagen solutions which precipitate at body temperature, thus leading to the formation of fibers which remain at the injection site whereas the excipient is progressively resorbed. U. S. Pat. No. 5,658,593 in one embodiment provides micro capsules based on atelocollagen optionally mixed with a glycosaminoglycan such as chondroitin-4-sulfate, the micro capsules containing granules of hydroxyapatite in suspension in a viscous bio compatible carrier

solution of a gel of atelocollagen optionally mixed with a glycosaminoglycan, in particular chondroitin-4-sulfate, for use as a filler material in forming injectable implants.

U. S. Pat. No. 5,626,861 teaches a method for the fabrication of three-dimensional macro porous polymer matrices for use as bone graft or implant material was developed. The composites are formed from a mixture of biodegradable, bio compatible polymer and hydroxyapatite (HA), a particulate calcium phosphate ceramic. The method leaves irregular pores in the composite between 100 and 250 microns in size by formation of a solid gel comprising a soluble material and dissolving the material to form voids in the gel. In a preferred embodiment, implants are composed of a 50:50 poly(lactide-co-glycolide) (PLGA) polymer and reinforced by hydroxyapatite. Mechanical and histological analysis showed the matrix fabricated by this method to be structurally and mechanically similar to cancellous bone. Prior to degradation, pure polymer specimens exhibited an elastic modulus of 293 MPa and specimens which were 50% HA by weight exhibited a modulus of 1459 MPa. After six weeks of degradation under physiological conditions, the reinforcing effect of ceramic loading had diminished. Modulus of polymer matrices at all HA load levels had decreased sharply to approximately 10 MPa. Mean macro- and micro pore diameters of the polymer specimens were 100 mu m and 20 mu m respectively and remained constant throughout degradation. The implants are hardened, leached and then implanted into the subject where they are slowly degraded by natural bodily action over a period of time. The implant size and shape must be predetermined and thus may not perfectly fit the site to be repaired.

B. R. Constantz, *et als*, 1995, Skeletal Repair by in Situ Formation of the Mineral Phase of Bone, *Science*, **267**:1796-1799. Discloses a process for the surgical implantation of a paste that hardens in minutes under physiological conditions. The mixture comprises a mixture of calcium phosphates and sodium phosphate, and hardens due to the crystallization of dahlite, not mediated by a stimulus setting gel. The mixture

hardens regardless of whether it is placed in the body. The paste is a hydroxyapatite precursor and does not include gel components.

B. Flautre, *et als*, 1996, Evaluation of Hydroxyapatite Powder Coated with Collagen as an Injectable Bone Substitute: Microscopic Study in Rabbit, *J. Materials Science:Materials in Medicine*, 7:63-67, discloses an injectable mix of hydroxyapatite and collagen but there is no disclosure of providing a stimulus response setting material. The group uses HA and atecollegen and chondrotin-4-sulfate formulated as micro spheres, similar to the patents discussed above. There is no provision for a gel which forms in response to a stimulus provided by exposure to the environment of the body, and there is no provision for differential loss of materials to provide a porous matrix. A further study by the same group, G. Pasquier, *et als*, 1996, Injectable Percutaneous Bone Biomaterials: an Experimental Study in a Rabbit Model, *J. Materials Science:Materials in Medicine*, 7:683-690, discloses mixtures comprising an orthopaedic acrylic cement (polymethylmethacrylate ("PMMA")) and HA as well as HA and collagen. The PMMA was used as a reference bio-inert material. There is no disclosure of a stimulus setting gel for producing a composite which only hardens in response to a stimulus supplied by the body.

M. Ito, *et als*, 1994, Experimental Development of a Chitosan-bonded β-Tricalcium Phosphate Bone Filling Paste, *Bio-Medical Materials and Eng.*, 4:439-499, discloses a composite of chitosan and tricalcium phosphate containing alkaline oxides of calcium, magnesium or zinc, which provided the conditions to produce setting. Again the material hardens without regard to stimulus supplied from the body. A similar study is reported by M. Takechi, *et als*, 1996, Non-decay Type Fast-setting Calcium Phosphate Cement Using Chitosan, *J. Materials Science:Materials in Medicine*, 7:317-322. Takechi uses sodium alginate or chitosan as a water insoluble gel to protect calcium phosphate cements from decay during setting under physiological conditions. In these materials the cements set as the normally do and the gel forms in response to the calcium

provided by the cement. Again there is no gel formation in response to a physiological stimulus for the composite material.

There is a continuing and long felt need for alternative implant materials for the treatment of damage to bony tissues by injury or disease. The art has not heretofore provided a fluid or shapeable implant material which comprises both a bone growth supporting matrix such as a ceramic matrix, and a stimulus sensitive gel which can be shaped to fill an injury site and then hardens to support the injured tissue during healing. The art has not heretofore provided a polymer/ceramic composite suitable for use in bone repair wherein a stimuli sensitive gel is used as a fluid carrier to place a ceramic matrix into a damaged bony tissue wherein the gel hardens in response to a physiological condition such as temperature, pH, ionic strength and the like in the presence of the ceramic.

SUMMARY OF THE INVENTION

The present invention provides a composition which comprises a polymer or polymer solution that forms a gel under controlled parameters and a ceramic matrix, the composition being fluid under non-physiological conditions and non fluid under physiological conditions. Polymers may be resorbable or nonresorbable, natural or synthetic and the solution aqueous or non aqueous. Preferred polymers are polysaccharides, polyamides or polyamino acids, however any polymer or polymer solution that is biologically compatible and that is fluid under nonphysiological conditions and increases in viscosity under physiological conditions is suitable. As used in this application, physiological conditions means conditions normally found in a mammalian body such as pH in the range of 4 to 9, ionic strength of around 0.15 or temperature in the range of 35-40°C. Stimuli sensitive gel means a natural or synthetic polymer that increases in viscosity, gels or crosslinks in response to a stimulus such as a change in

temperature, pH, ionic strength, light or the like. In contrast to the rigid composites synthetic grafts of the prior art, the compositions of the present invention may be injected at a trauma site, such as a fracture and shaped to fill any voids present, forming and in situ splint and scaffold for the growth of new bone. The composite may also serve as a controlled release device for a therapeutic agent such as a bone growth factor, an antibiotic, a chemotherapy drug, or a cytokine. The composites may include bone morphogenic proteins or other osteoconductive agents. Preferably the composites are formed in such a manner that the final solid implant is porous with macroscopic pores on the order of 100 to 200 microns in cross section. In an alternative embodiment a near net shape forming composition is employed wherein the polymer is a bio compatible shear thinning polymer that forms a gel under ambient pressure and a ceramic component carried therein. The shear thinning polymer is one that forms a gel in response to a stimulus such as ultrasonic vibration or injection.

Alternatively the invention may be viewed as a method of forming a solid implant in a mammalian body which comprises mixing a gel forming component with a ceramic forming component to provide a fluid mixture, placing the fluid mixture into a mammalian body wherein the fluid mixture gels after placement in the mammalian body in response to a stimulus provided by conditions present or induced in the mammalian body. Conditions present in the mammalian body includes normal body temperature, ionic strength, pH and the like. Conditions that can be induced in the body include ultrasonic vibration, externally applied magnetic fields, irradiation with from a radiation source or light or other electromagnetic radiation. Preferably the fluid mixture comprises a gel forming polymer, a calcium phosphate ceramic, and a soluble material which will produce voids in the final inplant, the voids having an average cross section in the range of 100 to 200 microns. The soluble material is preferably a second polymer which degrades or dissolves relatively rapidly under physiological conditions. Especially

preferred polymers dissolve by enzymatic action leaving non toxic, non irritating residues.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plot of the viscosity of hydroxyapatite (HAP) slurries with different polymers.

Figure 2 is a plot of solids loading effect on the viscosity of HAP in 0.4 wt% carrageenan.

Figure 3 is a plot of solids loading for three polysaccharide polymers.

Figure 4 is a plot of temperature against viscosity in HAP carrageenan supensions .

Figures 5a and 5b are Scanning Electron Micrographs showing the structures of 30wt% HAP in 2wt% carrageenan (a) and 40wt% HAP in 2wt% carrageenan (b).

Figure 6 is a plot of the dissolution of HAP/carrageenan and HAP powder at pH 6.5 in a simulated plasma serum.

Figure 7 is a plot of the fraction of carrageenan leached from the composition against time.

Figure 8 is a plot of the viscosity of chitosan/HAP suspensions as a function of HAP wt% in 0.1N acetic acid.

Figure 9 is a plot of the compression modulus of chitosan/HAP composites showing the effect of solids loading.

Figure 10 is a plot of the compression modulus of HMW chitosan/HAP and chitosan/TCP showing the effect of the ceramic calcium phosphate phase.

Figure 11 is a plot of the release of 5-fluorouricil from chitosan/HAP and chitosan/TCP compositions.

Figure 12 is a plot comparing release of 5 fluorouricil and bovine serum albumin from a chitosan/HAP composition.

DETAILED DESCRIPTION OF THE INVENTION

Mechanisms by which bone may fail include brittle fracture from impact loading and fatigue from constant or cyclic stress. Stresses may act in tension, compression, or shear along one or more of the axes of the bone. A synthetic bone substitute must resist failure by any of these stresses at their physiological levels. A factor of safety on the strength of the implant may ensure that the implant will be structurally sound when subject to hyper physiological stresses. The solid implants of the prior art all require the injured bone to conform to the shape of the implant, requiring invasive surgery, long recovery times, fixation devices inserted into the bone, rigid external or internal splints and ingrowth of bone into the implant ("knitting"). Especially in the elderly, implants may fail due to failure of the bone regrowth and therefore failure of the implant to become joined to the bone. The rigid implant of U. S. Pat. No. 5,626,861 exemplifies the traditional approach of forming the implant ex vivo followed by surgical insertion into the injured bone.

In contrast to the prior art approach, it has now been found that stimuli sensitive gels can be combined with bone precursors such as hydroxyapatite ceramic particles to permit placement of fluid materials into damaged bone. The fluid mixtures form rigid structures on exposure to physiological conditions forming the implant as an integral part of the bony structure. The hardening of the implant may be triggered by any stimuli that can be provided directly or indirectly under physiological conditions. Examples of direct stimuli include temperature, pH, ionic strength, and the like, as they occur in a mammalian body. Examples of indirect stimuli that may be applied under physiological conditions include external heating or cooling, light and other electromagnetic radiation across the broad range of the spectrum, magnetic fields, induced or applied electrical charge or current, and the like.

A variety of bio compatible polymers can be used. The stimulus sensitive polymer may be any bio compatible polymer or copolymer which forms a gel or crosslinked structure in response to a stimulus which may include temperature, pH, ionic strength, solvent composition, sheer stress, light, and the like or a combination of these factors. Preferred polymers are described in co-pending application Serial No 08/870,368; Filed June 6, 1997 incorporated herein by reference. Preferred stimulus sensitive polymers are random copolymers of a [meth-]acrylamide derivative and a hydrophilic comonomer, wherein the random copolymer is un the form of a plurality of linear chains having a plurality of molecular weights greater than or equal to a minimum gelling molecular weight cutoff.

The [meth-]acrylamide derivative is an N, N′-alkyl substituted [meth-]acrylamide including but not limited to N-isopropyl[meth-]acrylamide, N,N′-diethyl[meth-]acrylamide, N-[meth-]acryloylpyrrolidine, N-ethyl[meth-]acrylamide, and combinations thereof.

The hydrophilic co monomer is any hydrophilic co monomer that polymerizes with the [meth]acrylamide derivative to produce a bio compatible polymer. Preferred hydrophilic co monomers are hydrophilic [meth-]acryl- compounds such as carboxylic acids, [meth-] acrylamide derivatives, and the [meth-]acrylamide esters. The preferred aqueous solvent is deionized water, the solvent may also contain salts. Any biologically acceptable nonaqueous solvent that dissolves the polymer may also be used. In addition to the non-resorbable gelling copolymer of N-isopropyl(meth-)acrylamide and (meth)acrylic acid a biodegradable (resorbable) copolymer exhibiting similar gelation properties may also be used in polymer/ceramic composites that gel in response to physiological conditions. The biodegradable (resorbable) thermally gelling copolymer is obtained by grafting of the oligo(meth-)acrylamide derivative side chains on a biodegradable polymer backbone. Examples of the suitable biodegradable polymers

include, polysaccharides and poly(aminoacids). The preferred biodegradable polymers are degraded by enzymatic hydrolysis to non toxic, non irritating residues.

Other polymers include the polysaccharides such as chitosan. Chitosans provide an additional new biodegradable component of polymer-ceramic composites suitable for injectable, resorbable templates for bone tissue regeneration. The rationale of using chitosans for this purpose is based on the fact that chitosan solutions gel in response to pH change from slightly acidic to physiological.

The unique aspect of this novel system is that at pH lower than 6.5 the chitosan-ceramic suspension is a paste-like flowable system and at physiological pH the polymer undergoes a phase transition resulting in entrapment of ceramic component within the reversible gel matrix. Thus, application of pH-reversible gels enables the creation of an implant which may be tailored to any shape of a bone defect needed to be filled. In contrast to the rapid setting gel/cement compositions which harden without regard to whether they are placed into the physiological environment, the present invention hardens on exposure to the physiological conditions that occur only after they are placed into the body. As a consequence, the stimulus response compositions of the invention and practice of the method of the present invention provides a bone filling biomaterial which can be pre-mixed and placed in stages following a sterilization or other treatment without premature setting.

Other polysaccharides such as xanthin gum, (available from Kelco), locust bean gum (available from Aldrich), and carrageenan (available from Aldrich, mainly K-carrageenan) are also useful in the invention.

Calcium phosphate ceramics are preferred for use as the ceramic component of implants in the repair of bone defects because these materials are non-toxic, non-immunogenic, and are composed of calcium and phosphate ions, the main constituents of bone. Both tricalcium phosphate (TCP) [$Ca_3(PO_4)_2$] and hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] have been widely studied for this reason. Calcium phosphate

implants may be osteoconductive, and have the apparent ability to become directly bonded to bone. As a result, a strong bone-implant interface can be created. However, the mechanical properties of calcium phosphate ceramics make them ill-suited to serve as a structural element. Ceramics are brittle and have low resistance to impact loading. For this reason the ceramic component is combined with a polymeric component which adds elastic strength to the composition overcoming the shortcomings of the ceramic alone while retaining its positive features.

Other useful ceramics include other calcium or magnesium phosphates, aluminas, and the like. Any nontoxic, non immunogenic ceramic may be substituted for calcium phosphate in special circumstances such as an application wherein resporbtion of the ceramic component is not desired. Calcium phosphate ceramics have a degree of bioresorbability which is governed by their chemistry and material structure. High density HA and TCP implants exhibit little resorption, while porous ones are more easily broken down by dissolution in body fluids and resorbed by phagocytosis. However, TCP degrades more quickly than HA structures of the same porosity in vitro. In fact, HA is relatively insoluble in aqueous environments.

These solubility differences permit the use of mixed calcium phosphate ceramics to control the final structure of the implant by using for example TCP particles sized to be selectively dissolved by bodily fluids and provide voids in the final structure and HA particles sized to crystallize under physiological conditions to provide a mineral matrix to foster bone ingrowth into the implant. Preferably the voids will be in the range of 100 to 200 microns, the preferred size for supporting cell growth. Other non-toxic salts can be substituted for TCP for special purposes in forming voids in the implants. Implants having a macro porus structure which pores on the order of 100 to 200 microns are strongly preferred, although pores may be smaller as in 50 to 150 microns, larger as in 100 to 300 microns, or cover a broader range as in 50 to 500 microns and still provide useful implants.

Bone repaired with the use of a conventional polymeric implant such as those described in U. S. Patent 5,626,861 will be required to be immobilized for between six and eight weeks, the standard procedure for conventional fractures. Where the implants of the present invention permit less invasive surgery and in situ fixation, the healing time may be reduced.

All fractures are subject to static loading even while immobilized in a cast, i.e., there is a load resulting from the weight of the bone itself. In order for the implant to unite bone segments in a fracture, it must have initial strength sufficient to provide the stability necessary for healing to begin. Further, the resorting implant must retain a degree of strength throughout the bone remodeling cycle. Strength retention in the implant is governed by the degradation rate of the polymer in the polymer-ceramic composite. Both high strength retention over time and rapid weakening of the scaffold may be detrimental to the bone repair process. Slow implant resorption can shield immature skeletal tissue from the functional stresses necessary for complete remodeling. Conversely, rapid degradation may prematurely shift load bearing to the new bone and cause its collapse. Preparation of example implants is described below to illustrate the invention and not by way of limitation. The examples are not intended to limit the invention which is defined by the claims set out below.

Procedures and Sample Preparation

Xanthin gum solution was prepared by addition of the powder at room temperature with stirring into deionized water or sodium chloride solution with desired ionic strength. The resulting smooth solution was then allowed to stand overnight to release all air bubbles. Solutions of locust beam gum and carrageenan gum were prepared at 70-75°C and stirred until all powders dissolve.

Hydroxyapatite(HAP)-xanthin gum and HAP-locust bean gum pastes were prepared by mixing the desired amount of HAP powder (Aldrich) into the polysaccharide solution using either a magnetic stir plate (<30wt% HAP) or mechanic stirrer at higher solids loading (>30wt% HAP). To prepare the HAP-carrageenan paste, the carrageenan solution was maintained at 70-75°C in a water bath during mixing. Rheological measurements such as viscometry, strain sweep and oscillation were conducted using a Bohlin Rheometer.

HAP-polysaccharide paste samples prepared for mechanical test and dissolution experiment were crosslinked in simulated blood plasma electrolyte solution overnight at room temperature. The simulated blood plasma electrolyte solution contains 3mM KCl, 1.5mM MgCl2, 4.2mM NaHCO3, 1.0mM KH2PO4, 138mM NaCl and 2.5mMCaCl2. It was adjusted to pH 7.4 by the addition of NaOH solution before use.

HAP-polysaccharide paste samples for microstructure and porosity studies were prepared and crosslinked in simulated blood plasma electrolyte solution as described above. They were then cut into small rectangles and quickly frozen in liquid nitrogen, then dried in vacuum at -12°C for at least 24 hours.

HAP dissolution kinetics was measured using the constant composition method at 25°C. The in-vitro carrageenan leaching experiments were conducted in simulated blood plasma electrolyte solutions and concentration of leached carrageenan was determined by a colormetric method using methylene blue.

Example 1

Rheological Studies of Calcium Phosphate-Binder Suspensions

The viscosity of HAP suspensions containing one of the three binders selected was measured as a function of binder properties, binder concentration, HAP solids loading and temperature.

Viscosity is a transport property. It must be sufficiently low to facilitate suspension transfer to the mold. A shear thinning behavior is desired. This means that at very low shear rates suspended HAP particles remain stationary because the high viscosity of the binder solution is below the yield point. Higher shear rates that are encountered during pouring or on a vibration bed during filling can effectively reduce the viscosity. As shown in Figure 1, all three slurries containing 30wt% HAP showed shear thinning behavior. Among them, HAP suspension containing carrageenan has the lowest viscosity even though the carrageenan concentration is higher (0.4wt%) than the other two binders (0.25wt%).

Solids loading has a great influence on viscosity is illustrated in Figure 2. The viscosity of these systems is almost constant with HAP solids loading equal or below 30wt% as shown in Figure 3. Further increasing the solids loading to 40wt% dramatically increased the viscosity of the suspensions. As a matter of fact, the highest HAP loading achievable is about 43wt%. Above that it became difficult to measure rheological properties.

Effect of temperature on gelation properties has also been investigated. It is known that temperature up to 93°C has no effect on viscosity of xanthin gum solutions. Locust bean gum solution, prepared at 70-80°C, also showed no significant changes in viscosity as temperature decreases. K-carrageenan solution, prepared at 70-75°C in a similar way to locust bean gum, formed a rigid gel upon cooling. Its rheological behavior

was investigated as a function of temperature. Temperature dependent viscosity measurements of carrageenan were started at 70°C and eventually cooled to 20-30°C in a water jacket of the rheometer.

As shown in Figure 4 at higher temperature (50-70°C), the suspension containing 30wt% HAP and 2wt% carrageenan has low viscosity. It formed gel around 40°C, indicated by the sudden increase of viscosity. Below 40°C, the gel is fractured by applied shear force, resulting in the decrease of viscosity. A 2wt% carrageenan solution without HAP showed a similar temperature dependence (Figure 4). This thermal gelation property makes the HAP-carrageenan suspension a good candidate as self-setting bone filling materials. It was fluid-like and injectable above 45°C, and formed a rigid gel at body temperature of 37°C.

Example 2

Materials Characterization

The microstructure and porosity of HAP-carrageenan pastes were studied by scanning electron microscopy (SEM). These pastes were prepared at 70-75°C and cross linked in simulated plasma electrolyte solution as described above. They were then cut into small rectangular pieces with rough dimension of 2.5cm x 0.5cm x 0.3cm. To preserve structures, they were quickly frozen in liquid nitrogen, then dried in vacuum at -12°C for at least 24 hours. The dimension of each piece was measured using a caliber before and after freeze drying to check on the shrinkage. While the samples containing 30wt% or 40wt% HAP and 2wt% carrageenan showed less than 5% shrinkage, a sample containing 10wt% HAP and 2wt% carrageenan shrank 10-20%, indicating a structure collapse during freeze drying. The SEM images of 30wt% and 40wt% HAP in 2wt% carrageenan are shown in Figure 5. The structure of the 30wt% HAP contained HAP

agglomerates of 5-10 μm, and pores ranging from 5-15μm throughout the monolith. An increase of HAP loading to 40wt% produced denser material and reduced pore size to less than 5μm.

In Vitro Response Test

HAP dissolution by constant composition method

The kinetics of HAP dissolution of HAP-carrageenan pastes was studied by constant composition (CC) techniques. An under saturated solution containing 0.125mM $CaCl_2$ and 0.075mM KH_2PO_4 and 0.15mM NaCl was prepared by mixing stock solutions of 0.1M $CaCl_2$ and 0.1M KH_2PO_4 in 0.15M NaCl. The solution pH was adjusted to 6.5 using 0.1M KOH solution. Nitrogen saturated with water vapor was purged through the prepared solution to exclude carbon dioxide. A combination pH electrode (Corning) was used as a probe to monitor solution pH. An amount of 0.083g of 30wt% HAP-2wt% carrageenan paste was weighed and added to the under saturated solution to initiate the dissolution experiment. The solution pH was kept constant by the potentiostatic controlled addition of 0.30M NaCl and 0.007M HCl. The rate of dissolution was calculated from the rate of addition of the acid after correcting for the volumes required to maintain constant pH. For comparison, the dissolution rate of 0.025g HAP powder, same amount of HAP as in HAP-carrageenan, was measured using the same technique [Figure 6]. Our CC results indicate both samples have high initial rates, and slowed down as more and more calcium and phosphate ions accumulated in the solutions. HAP-carrageenan paste had a lower initial dissolution rate than the free HAP powders. As the carrageenan gradually leached out, the HAP-carrageenan paste became loosely attached and acted more like free particles.

Carrageenan leaching studies in simulated blood plasma electrolyte solutions

Suspensions containing 2wt% carrageenan and 0 to 40wt% HAP were prepared at 75°C. They were poured into small weighing dishes and formed blocks upon cooling to 37°C. After weighing each block (about 8 gram),each block was placed in 20 ml of simulated blood plasma solution at 37°C. Periodically, the solution was collected and replaced with new solution. The HAP powder that was remaining in the solution was separated by centrifugation. The clear solution samples were diluted and mixed with methylene blue. The concentrations of carrageenan were determined by measuring adsorption at 559nm against a standard calibration curve. The result is shown in Figure 7.

Among the paste samples tested, those containing less than 20wt% HAP were softened and broken down after 24 hours. Therefore, no data was available after this period. At higher HAP loading, the blocks maintained shape. The results indicate the higher the HAP solid loading, the slower carrageenan leached out.

In vivo Results

Implants prepared according to the above procedure were placed in an induced bone defect in mice and produced an acute adverse response. Thus while carrageenan is an attractive candidate material based on physical properties it was found to lack biological compatibility when formulated as a polymer/ceramic composite. The high dissolution rate of carrageenan probable contributes to the adverse response. Although the carrageenan illustrates the desireable physical properties of the invention, it is not useful in the method on the invention because it is unsuited to implantation in a mammalian body.

Example 3

Chitosan study

Methods

High molecular weight (HMW) chitosan, 1.1-1.6x10^6 D, was purchased from Aldrich Chemical Company, Inc., low molecular weight (LMW) chitosan, 70 KD, was purchased from Fluka Chemie. Chitosans were dissolved in 0.1N HCl and purified before use by precipitation into acetone/water mixture. Calcium phosphate HAP, tricalcium phosphate (TCP), Bovine serum albumin (BSA), 5-fluorouracil (5-FU), and lysozyme (hydrolytic enzyme) were purchased from Sigma Chemical Company, and used as received. Phosphate buffered saline (PBS), pH 7.4 was used to prepare all polymer-ceramic suspensions and as a release medium in BSA and 5-FU release experiments.

Preparation of chitosan-HAP and chitosan-TCP suspensions

Solutions containing 0.5-2 wt% of purified chitosan were prepared in 0.1N HCl. Chitosan-HAP or -TCP suspensions were prepare by simple mixing of the ceramic component with chitosan solution. Suspensions containing 20-45 wt% of HAP and 30-50 wt% of TCP were prepared.

Compressive modulus measurements

Experiments were performed using Dynamic Mechanical Analyzer equipped with the stainless-steel parallel plate measuring system. Compressive modulus was determined by using a uniaxial, unidirectional compressive deformation. The samples, in the form of cylindrical disks, were held in place initially with a minimal static stress, then the static

stress was increased. The response of the sample (static strain) was used to calculate the static compressive modulus.

Release of model compounds

The release of 5-FU and BSA from chitosan-HAP or -TCP composites was conducted in PBS, pH 7.4 at 37°C. A cylinder shaped samples of the composites were cured in a small diameter dialysis tubing. The amount of released compounds was determined by monitoring UV absorption of the release medium at 266 and 280 nm for the 5-FU and BSA, respectively. To maintain sink conditions, samples were transferred into fresh release medium at predetermined time intervals. The release kinetics data were reported as fraction of the total amount released versus time.

Results

In order to obtain chitosan-calcium phosphate composites suitable for the injectable, resorbable scaffolds for bone tissue regeneration, several properties of the composites need to be investigated and optimized. The most important requirements are the following: bio compatibility of the materials and their degradation products, injectability of the polymer-ceramic suspensions, suitable gelation kinetics of the composites, controlled degradation rates, ability to release small and large therapeutic agents and finally osteoconductivity. As a consequence of the above requirements, we have concentrated on investigation and optimization of the following properties: rheological properties of the suspensions, and water content, porosity, compressive strength, and degradation rate of the cured composites. The ability of the composites to release therapeutic agents was also evaluated.

Chitosan-calcium phosphate suspensions: preparation and rheological properties

Chitosan dissolves in diluted acids at pH 2-3 but the pH of the obtained solution may be titrated up to a pH of 6 with no change in polymer solubility. Therefore, chitosan-ceramic suspensions may be prepared at a wider pH range, i.e. 2-6. The pH of chitosan solution is important since it affects the solubility of the calcium phosphate phase of the composite.

Rheological properties of chitosan solutions and chitosan-calcium phosphate suspensions were investigated as a function of chitosan concentration, molecular weight, content and type of a calcium phosphate phase (HAP or TCP). As illustrated in Figure 8, chitosan-HAP suspensions containing up to 40 wt% of HAP phase exhibited viscosities and flow suitable for injectable bone paste applications.

Compressive modulus

Compressive modulus is a measure of the resistance of the sample to compression and is an indicator of stiffness or rigidity. Relative compression moduli were determined asses the changes in mechanical properties of the polymer-ceramic composites resulting form the gelation of the polymeric component. The compressive moduli were determined for a series of polymer-ceramic composites differing in composition in terms of ceramic (HAP or TCP) as well as polymeric components (LMW or HMW chitosan). Results are presented in Figures 9 and 10. The effect of solids loading, i.e., the amount of ceramic phase in the composite, on the compressive modulus of HMW chitosan-HAP composite is illustrated in Figures 9 and 10. Composites with higher solids loading, 40 wt%, demonstrated higher relative compressive modulus as demonstrated by a steeper slope of the stress strain curve. A similar trend was observed for the chitosan TCP composites. The effect of the ceramic phase type is illustrated in Figure 10. Composites

containing HAP demonstrated higher relative compressive moduli than composites containing TCP. The trend was similar for both chitosans HMW and LMW (results not shown). The results of compressive modulus studies clearly demonstrated that the chitosan-calcium phosphate composites in a gelled state maintain mechanical integrity in contrast to the flowable, injectable properties of the corresponding suspensions .

Release of model compounds

Different therapeutic and osteoconductive agents may be incorporated into the thermally reversible polymer-ceramic composites as needed. Examples of therapeutic agents include antibiotics for the local treatment of possible infections, anticancer agents for the site specific treatment of bone tumors. Examples of osteoconductive agents include growth factors and bone morphogenic proteins. In order to asses the possibility of using our novel polymer-ceramic composites as delivery vehicles for therapeutic and/or osteoconductive agents we have investigated the release of model low and high molecular weight compounds.

An anticancer agent, 5-fluorouracil (5-FU) was used as a model low molecular weight compound and bovine serum albumin (BSA) was used as a model macromolecule. The results of the release experiments are presented in Figures 11 and 12. In Figure 11 the effect of two calcium phosphate ceramic phases are compared, showing a small rate advantage to HAP over TCP. As illustrated in Table 12 the release of 5-FU and BSA demonstrated different kinetics. While 5-FU released from the chitosan-TCP composite within 10 hours, BSA release was much less rapid and lasted for 40 hours. These results may be explained by the differences in the effective size of the releasing molecules. BSA, being a macromolecule demonstrated slower release kinetics due to the lower effective diffusion coefficient within the polymer-ceramic matrix. The release results

demonstrated that chitosan-calcium phosphate composites may be used as a matrix for the release of therapeutic agents and proteins such as growth factors and bone morphogenic proteins.

CLAIMS:

We claim:

1. A composition, comprising at least two phases: a biologically compatible polymer or polymer solution that forms a gel under physiologically controlled parameters and a biologically compatible ceramic component; the composition being fluid under non-physiological conditions and non fluid under physiological conditions.

2. A composition according to claim 1, wherein the polymer or polymer solution is selected from the group consisting or resorbable polymers, non resorbable polymers, natural polymers, synthetic polymers and the aqueous or non aqueous solutions thereof and combinations thereof.

3. A composition according to claim 1, wherein the polymer is selected from the group consisting of poly saccharides, polyamides, polyamino acids and combinations thereof.

4. A composition according to claim 1 wherein the polymer forms a gel on exposure to a pH in the range of 4 to 9.

5. A composition according to claim 1 wherein the polymer forms a gel on exposure to a temperature in the range of 35 to 40°C.

6. A composition according to claim 1 wherein the polymer forms a gel on exposure to an ionic strength of about 0.15.

7. A composition according to claim 1 which comprises a therapeutic agent.

8. A composition according to claim 7 wherein the therapeutic agent is selected from the group consisting of bone growth factors, antibiotics, chemotherapy drugs, radioisotopes, osteoconductive factors, bone morphogenic proteins and cytokines.

9. A composition according to claim 1 which comprises a hydroxyapatite ceramic, or a tricalcium phosphate ceramic or mixtures thereof.

10. A composition according to claim 9 which comprises chitosan, xanthin gum, locust bean gum.

11. A composition according to claim 1 which comprises voids.

12. A composition according to claim 11 wherein the voids have a cross section in the range of 100 to 200 microns.

13. A method of forming a solid implant in a mammalian body which comprises the steps of mixing a biologically compatable gel forming component with a ceramic or a ceramic forming component providing a fluid mixture, and placing the fluid mixture into a mammalian body wherein the fluid mixture gels after placement in the mammalian body in response to a stimulus provided by conditions present or induced in the mammalian body.

14. A method according to claim 13 wherein the polymer is selected from the group consisting of resorbable polymers, non resorbable polymers, natural polymers and synthetic polymers and the aqueous or non aqueous solutions thereof.

15. A method according to claim 14 wherein the polymer is selected from the group consisting of polysaccharides, polyamides or polyamino acids.

16. A method according to claim 13 wherein the polymer forms a gel on exposure to a pH in the range of 4 to 9.

17. A method according to claim 13 wherein the polymer forms a gel on exposure to a temperature in the range of 35 to 40°C.

18. A method according to claim 13 wherein the polymer forms a gel on exposure to an ionic strength of about 0.15

19. A method according to claim 13 which comprises the step of mixing a therapeutic agent with the fluid mixture.

20. A method according to claim 19 wherein the therapeutic agent is selected from the group consisting of bone growth factors, antibiotics, chemotherapy drugs, radioisotopes, osteoconductive factors, bone morphogenic proteins and cytokines.

21. A method according to claim 13 which comprises the further step of mixing a material into the fluid mixture which produces voids in the gelled fluid mixture.

22. A method according to claim 13 wherein the material produces voids having a cross section on the order of 100 to 200 microns.

ABSTRACT

The present invention provides a composition which comprises a polymer or polymer solution that forms a gel under controlled parameters and a ceramic matrix, the composition being fluid under non-physiological conditions and non fluid under physiological conditions. Polymers may be resorbable or non resorbable, natural or synthetic and the solution aqueous or non aqueous. Preferred polymers are poly saccharides, polyamides or polyamino acids, however any polymer or polymer solution that is biologically compatible and that is fluid under nonphysiological conditions and increases in viscosity under physiological conditions is suitable. As used in this application, physiological conditions means conditions normally found in a mammalian body such as pH in the range of 4 to 9, ionic strength of around 0.15 or temperature in the range of 35-40°C. Stimuli sensitive gel means a natural or synthetic polymer that increases in viscosity, gels or crosslinks in response to a stimulus such as a change in temperature, pH, ionic strength, light or the like. In contrast to the rigid composites synthetic grafts of the prior art, the compositions of the present invention may be injected at a trauma site, such as a fracture and shaped to fill any voids present, forming and in situ splint and scaffold for the growth of new bone. The composition may also serve as a controlled release device for a therapeutic agent such as a bone growth factor, an antibiotic, a chemotherapy drug, a radioisotope, or a cytokine. The compositions may include bone morphogenic proteins or other osteoconductive agents. Preferably the compositions are formed in such a manner that the final solid implant is porous with macroscopic pores on the order of 100 to 200 microns in cross section. Alternatively the invention may be viewed as a method of forming a solid implant in a mammalian body which comprises mixing a gel forming component with a ceramic forming component to provide a fluid mixture, placing the fluid mixture into a mammalian body under conditions that cause the fluid mixture to increase in viscosity after placement in the body. Preferably the fluid mixture comprises a gel forming polymer, a calcium

phosphate, and a soluble material which will produce voids in the final implant having an average cross section in the range of 100 to 200 microns.

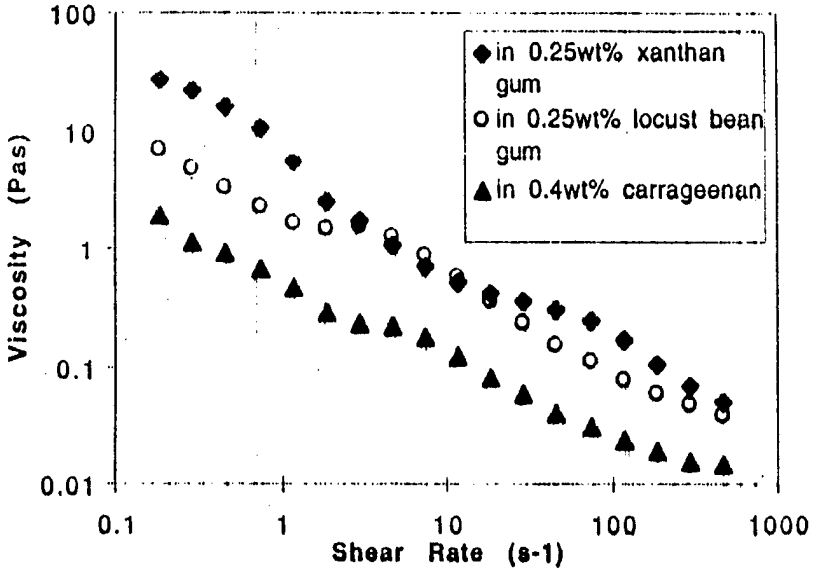
100
10
1
0.1
0.01
Viscosity (Pas)
0.1
1
10
100
1000
Shear Rate (s-1)
in 0.25wt% xanthan gum
in 0.25wt% locust bean gum
in 0.4wt% carrageenan

Figure 1

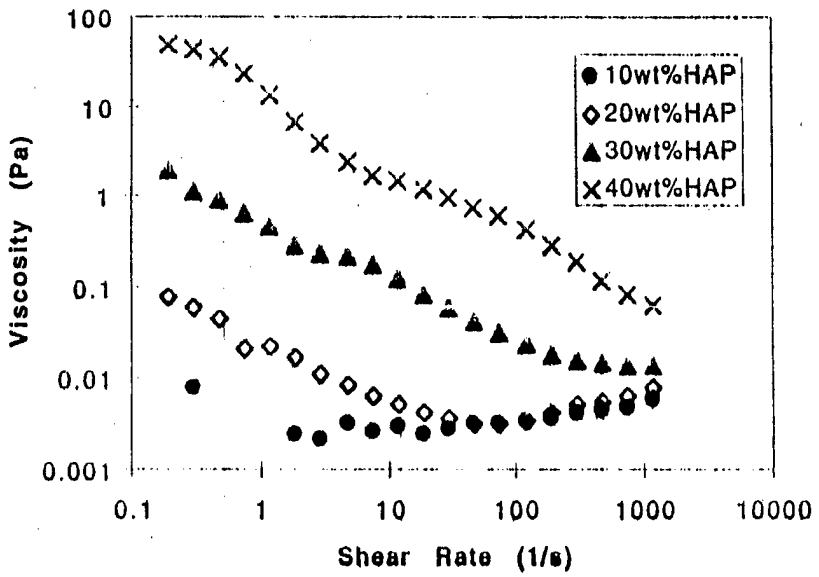
100
10
1
0.1
0.01
0.001
Viscosity (Pa)
0.1
1
10
100
1000
10000
Shear Rate (1/s)
10wt%HAP
20wt%HAP
30wt%HAP
40wt%HAP

Figure 2

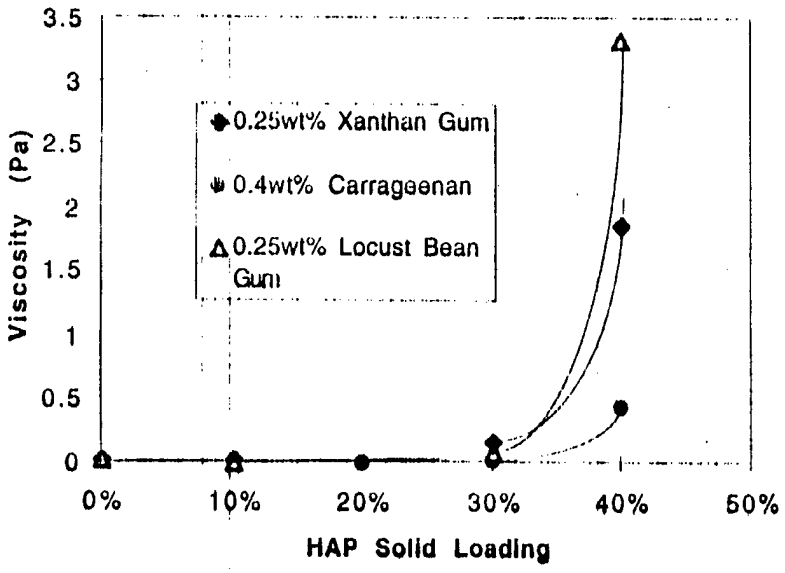
3.5
3
2.5
2
1.5
1
0.5
0
Viscosity (Pa)
0.25wt% Xanthan Gum
0.4wt% Carrageenan
0.25wt% Locust Bean Gum
0%
10%
20%
30%
40%
50%
HAP Solid Loading

Figure 3

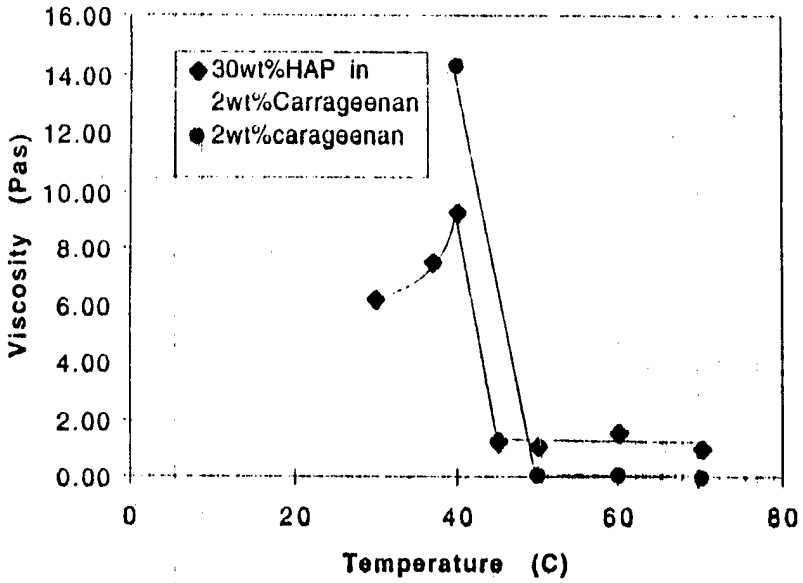
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Viscosity (Pas)
30wt%HAP in 2wt%Carrageenan
2wt%carageenan
0
20
40
60
80
Temperature (C)

Figure 4

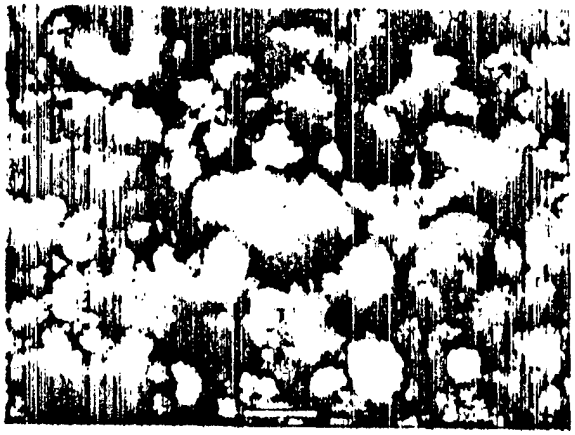

Figure 5a

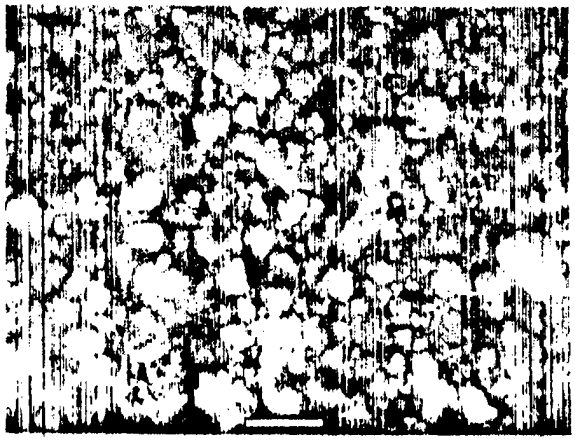

Figure 5b

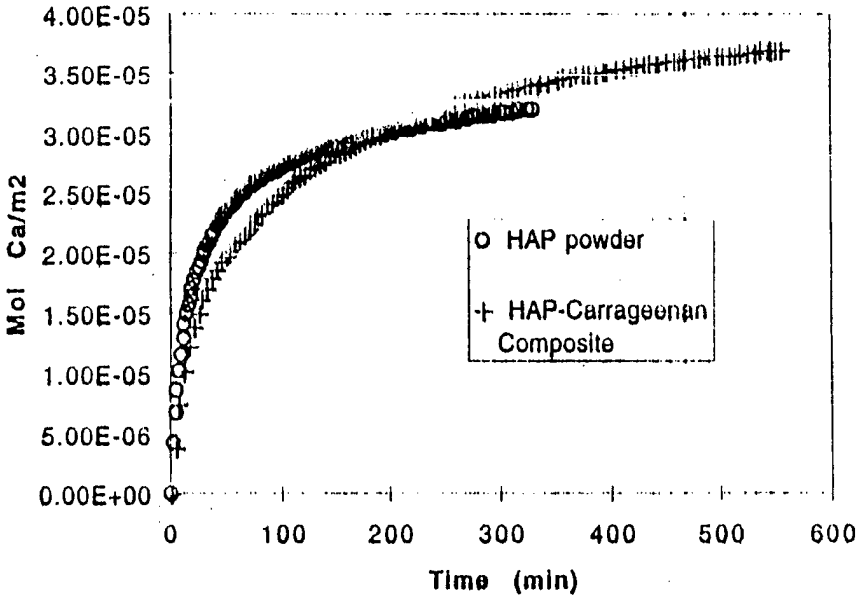
4.00E-05
3.50E-05
3.00E-05
2.50E-05
2.00E-05
1.50E-05
1.00E-05
5.00E-06
0.00E+00
Mol Ca/m2
0
100
200
300
400
500
600
Time (min)
HAP powder
HAP-Carrageenan Composite

Figure 6

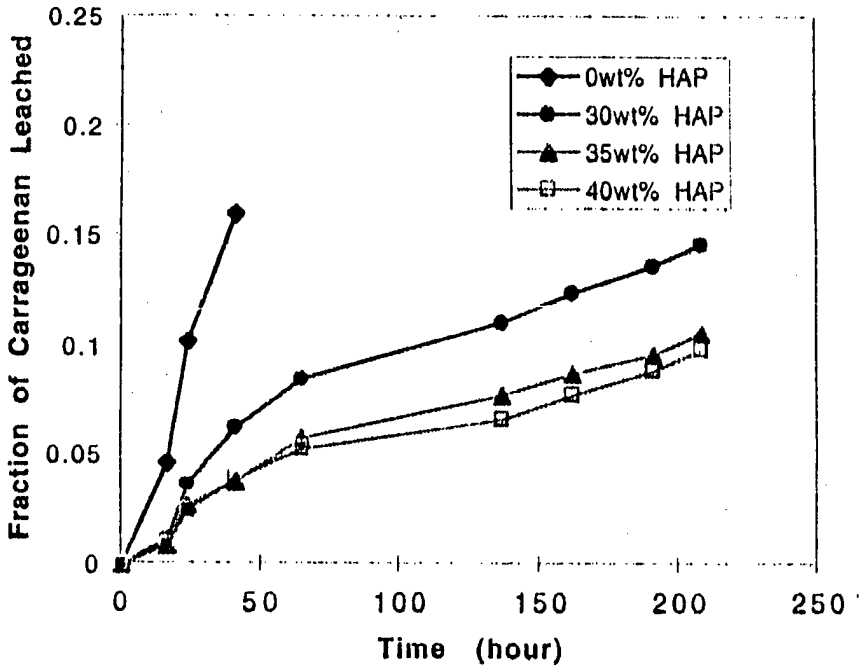
0.25
0.2
0.15
0.1
0.05
0
Fraction of Carrageenan Leached
0wt% HAP
30wt% HAP
35wt% HAP
40wt% HAP
0
50
100
150
200
250
Time (hour)

Figure 7

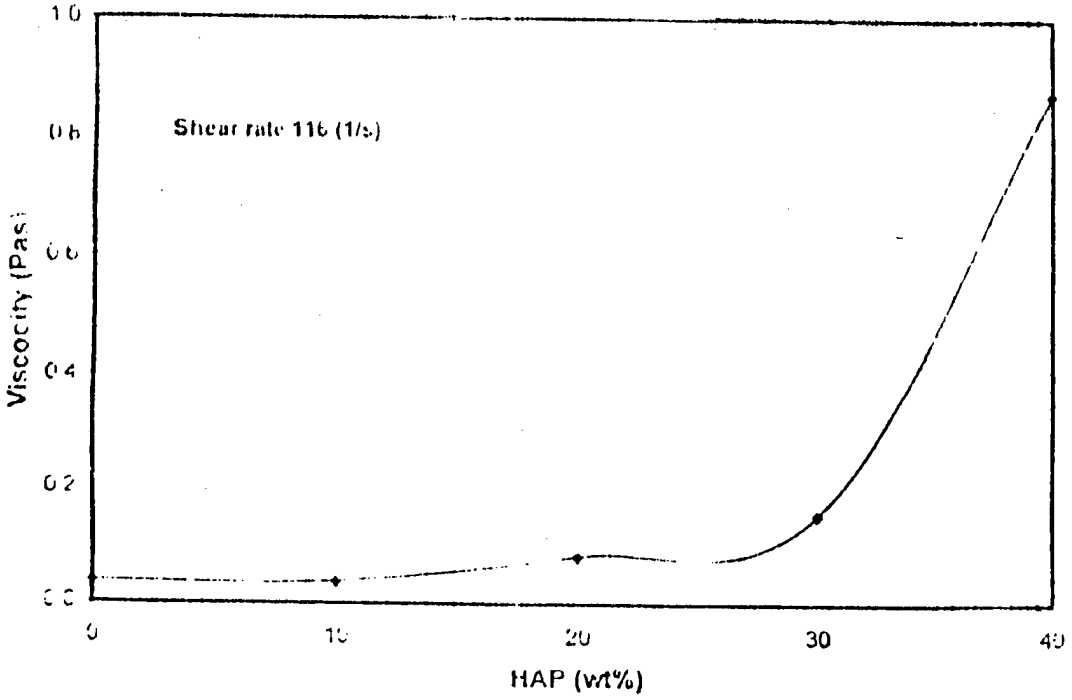

Figure 8

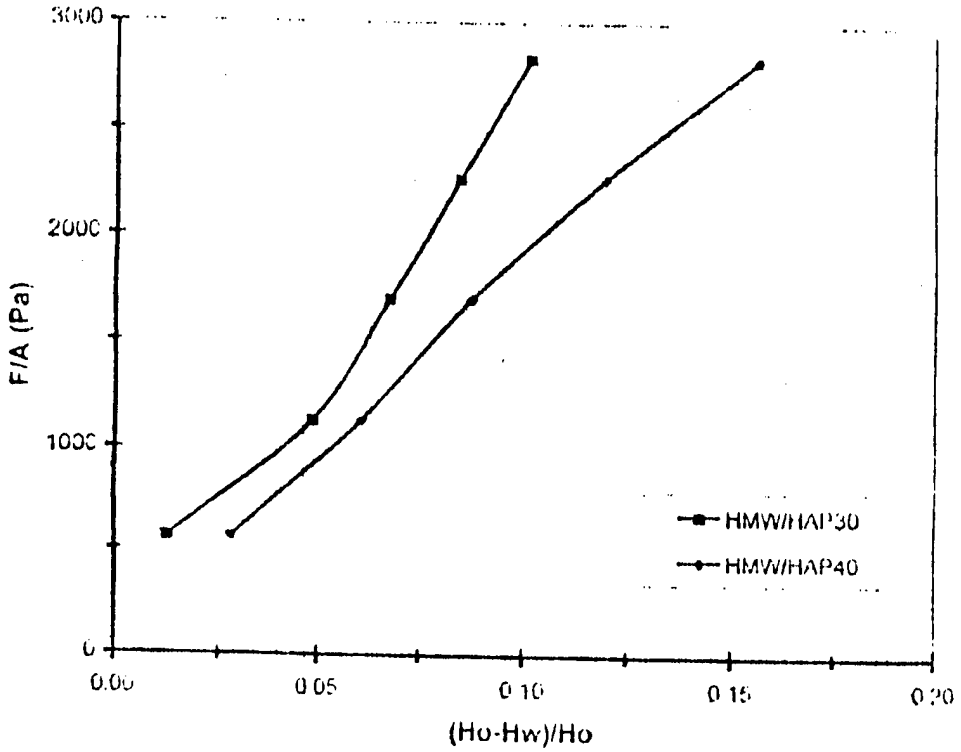
3000
2000
1000
0
F/A (Pa)
0.00
0.05
0.10
0.15
0.20
(Ho-Hw)/Ho
HMW/HAP30
HMW/HAP40

Figure 9

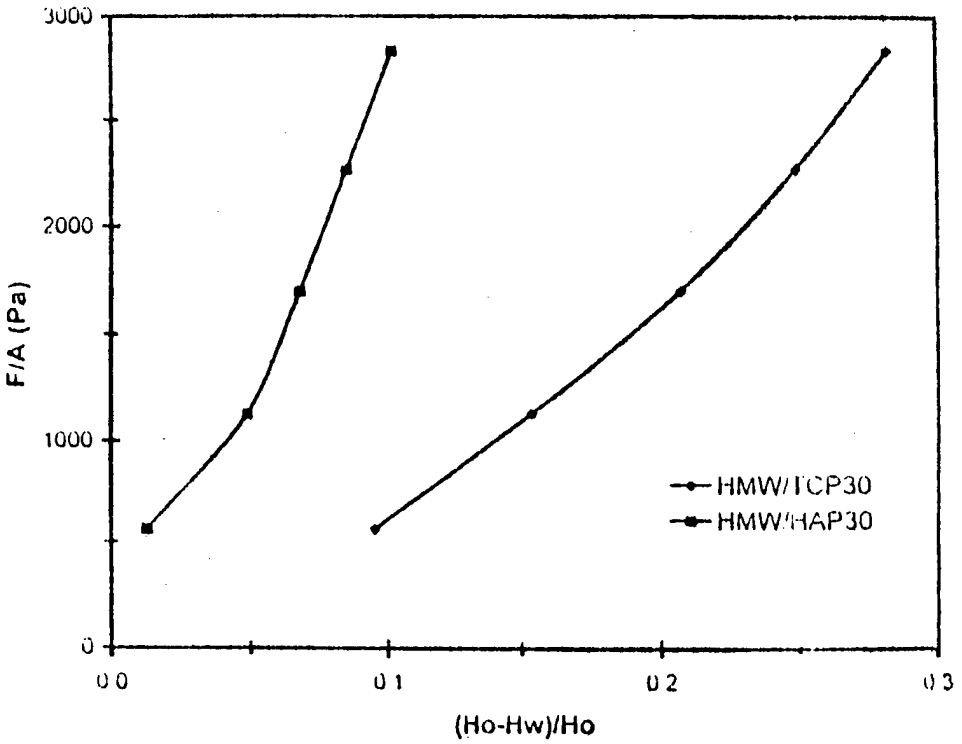

Figure 10

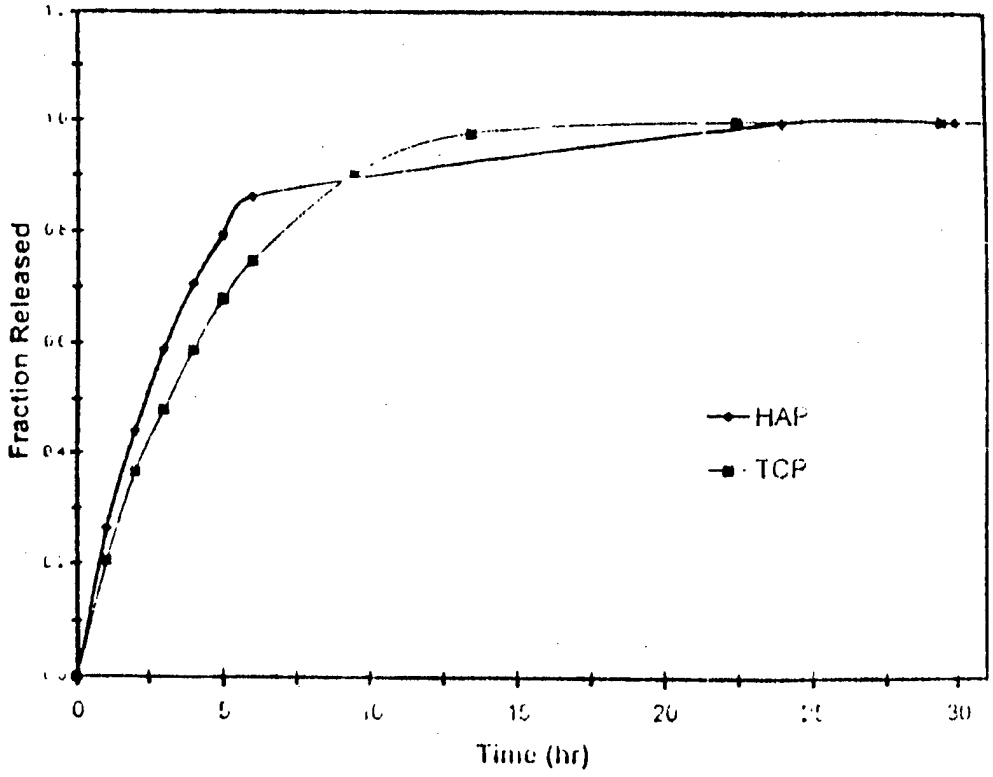

Figure 11

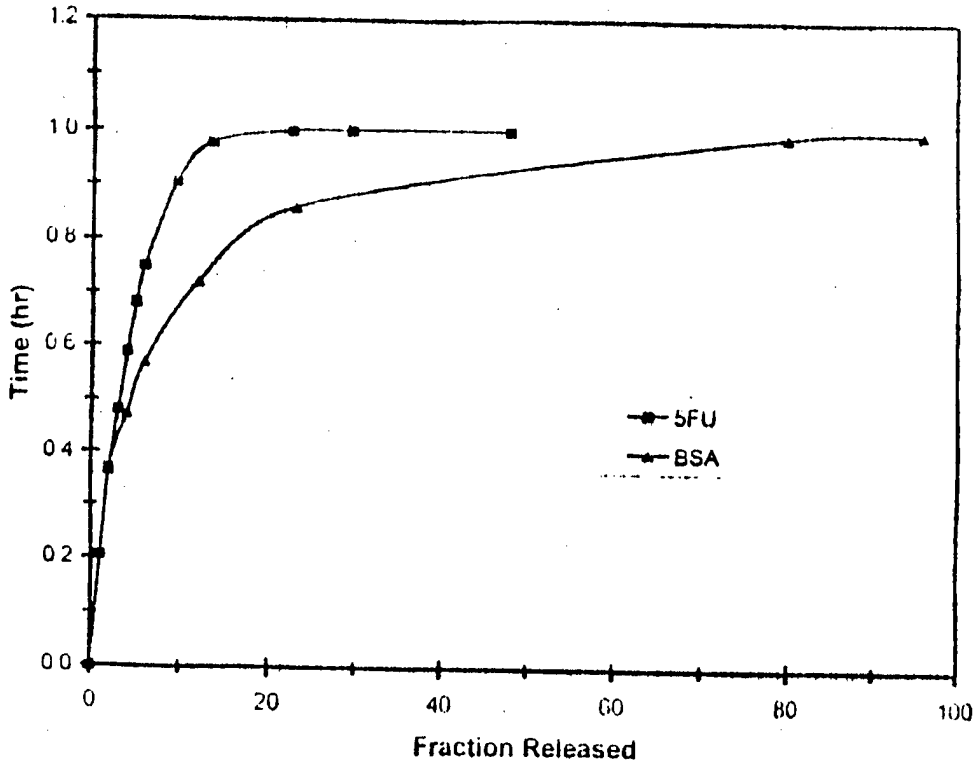
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Time (hr)
0
20
40
60
80
100
Fraction Released
5FU
BSA

Figure 12

Injectable, stimuli-sensitive polymer-ceramic composites

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ABSTRACT

For the treatment of bone loss due to trauma or disease, calcium phosphate bioceramic materials, such as hydroxyapatite, tricalcium phosphate and Bioglass®, have been used as synthetic bone grafts/fillers. Traditionally, these materials have been used in either granular or block forms. While the use of granular particles enable the surgeon to pack the defect, the problem with using granular bone filling materials is related to insufficient bonding between the particles resulting in displacement of particles from the bone defect by the flowing blood. Another problem relates to the large mismatch in the mechanical properties of the block and granular grafts and the natural bone surrounding the graft. In order to overcome these difficulties, the Pacific Northwest National Laboratory (PNNL) has developed novel polymer/ceramic formulations based upon stimuli sensitive polymers and bioceramic powders. These new polymer/ceramic composites will 1) prevent particle displacement while providing a biologically active materials for tissue regeneration, 2) improve the mechanical properties of the material over the ceramic materials, and 3) be easily placed by the attending physician due to the injectability and/or modability of the material. The polymers were from the chitosan family of polymers. Chitosan is a nontoxic, biodegradable natural polymer which has been reported to possess homeostatic properties as well as the ability to promote the bone regeneration by chitosan derivatives. The ceramic materials investigated include hydroxyapatite (HAP) and tricalcium phosphate (TCP). Composite formulations were evaluated for microstructural, mechanical, and rheological properties.

INTRODUCTION

Currently, large bone defects are repaired using three general approaches: autografts, allografts or synthetic grafts.[1] Autografts, where the donor and recipient are the same individual, have several disadvantages such as, limited supply, increased operative time, and donor site pain. Allografts, where donor and recipient are different individuals, present their own limitations including graft rejection, immunological concerns, and the concern over disease transmission from donor to recipient. For these reasons, synthetic grafts that posses the proper biocompatible and mechanical properties have been investigated and used as artificial graft materials.

In order for synthetic bone graft materials to be of value, they must have the following basic requirements:1) excellent biocompatibility, 2) proper mechanical properties, 3) the ability to be resorbed, and 4) ease of handling. Currently, there several types and formulations used as synthetic bone graft materials. These are predominately composed of calcium phosphate minerals such as tetracalcium phosphate (TTCP), tricalcium phosphate (TCP), hydroxyapatite (HAP) and Bioglass® (BG), a synthetic glass material. Table 1 lists the specific characteristics of each material. Hydroxyapatite and tricalcium phosphate are used widely as bone replacements in the fields of oral and plastic surgery.[2] HAP is classified as a bioactive ceramic because it develops chemical bonds with the surrounding tissue and facilitates the attachment of the implant to host tissues. TCP is classified as a resorbable bioceramic because it is gradually resorbed or dissolved and

replaced by natural tissue. Bioglass® is a class of unique synthetic materials that react in the presence of body fluids, whereby they enhance the body's ability to regenerate tissue and heal itself. This is accomplished by attracting essential biological elements produced by the body for healing and holding them in the wound or defect site while natural healing occurs. Bioglass® can be processed into many forms but is most commonly produced as granules or a fine powder used alone or combined with other materials. In addition, the physical form of these materials is either as a 3-dimensional block or in granular powders. The problem associated with these physical states is the difficulty in sizing the implant to the defect sites in order to get a tight fit (blocks) or the migration of particles away from the implant site (granules).

In an attempt to overcome these handling difficulties, calcium phosphate materials have been used in combination with polymeric components to form an injectable polymer/ceramic composite suitable for bone filling applications at low or non-load bearing sites.

In this paper, we report on the developed of novel polymer/calcium phosphate composites that are based upon stimuli sensitive polymers. The composite materials are designed such that their properties allow the composite to be used as a flowable or moldable paste (i.e. the resulting paste must have shear thinning characteristics). The paste can then be injected or placed into the defect site and the polymer gelled using the physiological conditions of the body as the stimuli. Another design feature will be that the mechanism of polymer gelation is caused by physiological conditions (such as pH). Therefore once

these paste is implanted, changes in pH or temperature will cause the polymer matrix to form a firm structure that conforms to the shape of the defect and prevents particulate migration.

Chitosan is a reversible stimuli sensitive polymer that gels as a result of pH changes. Chitosan is a member of a class of (1,4)-linked 2-amino-2-deoxy-D-glucans, a family of biodegradable and biocompatible cationic polysaccharides produced commercially by the partial deacetylation of chitin. Solutions of chitosan exhibit a liquid-gel transition around pH 7and therefore make an ideal polymer candidate for use in bone graft composites based upon stimuli sensitive polymers. Recently, chitosans have been investigated for many diverse medical applications such as wound dressings, contact lenses and materials for cell encapsulation. [3,4] Chitosan, having a chemical structure similar to hyaluronic acid, has also been implicated as a wound healing agent. It has been shown that the treatment of various canine tissues with a chitosan solution resulted in the inhibition of fibroplasia and enhanced tissue regeneration [5]. Recently, Klokkevold et al. investigated the beneficial effect of chitosan on osteoblast differentiation and bone formation in vitro.[6] Their results strongly suggested that chitosan potentiates the differentiation of osteoprogenitor cells and may facilitate the formation of bone.

Ito et al. [7-9] recently described the application of a composite containing chitosan and hydroxyapatite granules as a bone filling paste. The new self-hardening paste was prepared by using a combination of chitosan, hydroxyapatite granules, zinc oxide and

calcium oxide. The setting kinetics and the physical form, however, make these pastes unsuitable for injectable formulations.

In this work, different chitosan formulations were investigated as a biodegradable component of polymer-ceramic composites suitable for injectable, resorbable templates for bone tissue regeneration. The rationale of using chitosan for this purpose is based on the fact that chitosan solutions gel in response to pH change from slightly acidic to physiological. The unique aspect of this novel system is that at pH lower than 6.5 the chitosan-ceramic suspension is a paste-like moldable system and at physiological pH the polymer undergoes a phase transition resulting in entrapment of ceramic component within the gel matrix.

In this paper calcium phosphate/chitosan composites where evaluated for their physical, mechanical and chemical properties. Specifically the rheological properties, water content, porosity, compressive strength, and *in vitro* degradation rate of the cured composites were studied. Additionally, the ability of the composites to release therapeutic agents was also evaluated.

MATERIALS AND METHODS

High molecular weight (HMW) chitosan, 1.1-1.6x10^6 D, 86.2 mol% deacetylation, was purchased from Aldrich Chemical Company, Inc. Low molecular weight (LMW) chitosan, 7x10^4 D, 89.7 mol% deacetylation, was purchased from Fluka Chemika-BioChemika. The chitosan was dissolved in 0.1N acetic acid and purified before use by

precipitation into 7:1 acetone/water mixture. Hydroxyapatite powder (HAP) was purchased from Aldrich, β-tricalcium phosphate (TCP) was obtained from BassTech International. Bovine serum albumin (BSA), 5-fluorouracil (5-FU), and lysozyme were purchased from Sigma Chemical Company, and used as received. Phosphate buffered saline (PBS), pH 7.4 was used to cure all polymer-ceramic composites and as a release medium in BSA and 5-FU release experiments.

Preparation of chitosan-calcium phosphate suspensions

Solutions containing 0.5-2.0 wt.% of purified chitosan were prepared in 0.1N acetic acid. The pH of all solutions was adjusted to 6-6.5 with 0.5 M sodium hydroxide. Chitosan-HAP or β-TCP suspensions were prepared by mixing of the ceramic component with chitosan solution using a magnetic stirrer. Suspensions containing 20-45 wt.% of HAP and 30-50 wt.% of TCP were prepared.

Rheological properties

The rheological properties of chitosan-calcium phosphate suspensions were investigated using a Bohlin Rheometer. Viscosities of suspensions containing 20-45 wt.% of HAP and 30-50 wt.% of TCP in a 2 wt.% chitosan solution were measured over a shear rate range of 10^{-1} to 10^{3} s^{-1} using a bob and cup system. Before the measurements, pH of all suspensions was adjusted to the range of 6-6.5.

Water content in cured composites

Chitosan-calcium phosphate suspensions (pastes) were cured in PBS at pH 7.4. Samples of cured composites, in the form of cylindrical disks (diameter 15 mm, thickness 3-4 mm) were cut out with a corkborer. Water content in the cured composites was determined gravimetrically and the following equation was used to calculate $W_{\%}$,percent of water in the sample:

$$W_{\%} = (W_w - W_d)/W_w \times 100\%$$

where W_w denotes the weight of the wet sample and W_d denotes the weight of that sample in a dried state.

Microstructure Analysis

Particle size of HAP and TCP powders was analyzed by scanning electron microscopy (SEM) and powders surface area was analyzed by the Brunauer, Emmett, Teller (BET) nitrogen adsorption method using a Quantachrome Autosorb Automated Gas Sorption System, Quantachrome Corp.

Microstructure of chitosan-calcium phosphate composites was investigated by field emission scanning electron microscopy (FE SEM) using LEO 982 electron microscope. Sample preparation involved curing of the paste in PBS, freezing in liquid nitrogen and subsequently freeze-drying at –12°C.

Compressive modulus

Compressive modulus was evaluated by studying -a uniaxial, unidirectional compressive deformation. Experiments were performed using a Dynamic Mechanical Analyzer equipped with the stainless-steel parallel plate measuring system. The samples, in the form of cylindrical disks (diameter 15 mm, thickness 3-4 mm), were held in place initially with a minimal static stress. Once the test was initiated then the static stress was increased. The response of the sample (static strain) was used to calculate the static compressive modulus. The stress-strain curve was obtained by plotting F/A vs (Ho-Hw)/Ho where, F denotes the force, A denotes the sample area, Ho denotes initial height of the sample and Hw denotes the height of the compressed sample.

The compressive modulus, E [Pa], was calculated as the slope of the linear region of the stress-strain curve.

Enzymatic degradation

Enzymatic degradation of chitosan/HAP or TCP composites (40 wt % of ceramic phase) was investigated using lysozyme as the hydrolytic enzyme. Small samples (cylindrical disks: diameter 15 mm, thickness 3-4 mm) of the cured composites were placed in PBS solution containing 1 mg/ml of lysozyme. The progress of composite degradation was evaluated based on sample weight change as a function of time according the following equation:

$$\% \text{ Degradation} = (W_o - W_t)/W_o \times 100\%$$

where W_o denotes the initial weight of the sample and W_t denotes the weight of the sample at time t .

Release of model therapeutic agents

Chitosan-HAP or β–TCP suspensions containing 40 wt% of ceramic phase were prepared as described above. To each suspension 2 wt% of 5-FU or BSA was added and mixed thoroughly. Cylindrical shaped samples of the composites were cured at pH 7.4 in a small diameter dialysis tubing. The release of 5-FU and BSA from cured composites was conducted in PBS, pH 7.4 at 37°C. The amount of released compounds was determined by monitoring UV absorption at 266 and 280 nm for 5-FU and BSA, respectively. To maintain sink conditions, samples were transferred into fresh release medium at predetermined time intervals. The release kinetics data were plotted as fraction released versus time.

RESULTS AND DISCUSSION

The requirements for such synthetic bone filling composites may be summarized as follows: 1) biocompatibility of the materials, 2) injectability of the polymer-ceramic suspensions, 3) suitable mechanical properties of the composites, 4) controlled degradation rates, 5) ability to release therapeutic agents and, 6) osteoconductivity. The stimuli sensitive chitosan/calcium phosphate composites studied in this work address the issues of ease of handling, biocompatibility, resorption, delivery of therapeutics

mechanical properties. Issues relating to osteoconduction are not within the scope of this paper.

Biocompatibility of the composites was assumed not to be a huge issue since all of the materials have proven to be biocompatible. In order to have an injectible formulation, shear thinning characteristics are required. Therefore, the rheological properties of suspensions were evaluated. Composite degradation was studied to ensure that the composite formulations would not immediately disintegrate upon exposure to physiological environments. For the bone filler applications it is desirable that gelation of the chitosan/calcium phosphate composite occur with no significant volume change. In order to assess this characteristic, the water content of the polymers before and after gelation was measured. Finally the porosity, compressive strength, the ability of the composites to release therapeutic agents were evaluated.

Rheological and morphological properties of chitosan-calcium phosphate suspensions

To facilitate "injectability" of the bone filler composites, the material should flow easily through a small bore needle or syringe. This means that the viscosity of the suspension must be sufficiently low to facilitate suspension transfer through the needle to the wound site. In terms of rheological behavior, the composites need to demonstrate a Newtonian or shear thinning behavior, i.e., the viscosity is linearly dependent upon shear stress or decreases with increasing shear rate, respectively. However, once the material is injected at the wound site, the material should not flow away. To achieve this an immediate increase in viscosity is required. Thus, Newtonian behavior is not preferred, and shear

thinning behavior is desired. This means at very low shear rates suspended HAP particles remain stationary because of the high viscosity of binder solutions below the yield point. Figure ~~1A~~ 1B illustrates the shear thinning behavior of a chitosan/HAP composite containing 40wt % of the ceramic phase. As seen in this figure, the desired shear thinning behavior was observed: as the shear rate was increased, the viscosity of the ceramic/polymer composite decreased. Similar trends were seen for samples that contained TCP . However, chitosan/calcium phosphate suspensions containing, α-tricalcium phosphate (TCP) demonstrated lower viscosities at corresponding solids loading (40% by weight) (Fig 1A). It is believed that the difference in viscosities was related to the differences in particle size and shape, chemical composition, and surface morphology of the starting powders. Scanning electron micrographs (SEM) images showed that the HAP powder consisted of loosely structured agglomerates (composed of smaller HAP crystals) with a whisker-like surface morphology, ranging in size from 1 to 5 μm, as shown in Figure 2. BET measurements show a corresponding specific surface area of 61.2 m^2/g. The β-TCP, however, consisted of compact, smooth-surfaced particles ranging in size from 0.5-1.0 μm, and some 1-4 μm sized agglomerates, as shown in Figure 3. The specific surface area of β-TCP was measured at 1.529 m^2/g. The smaller particles present in the HAP composite most likely caused an increase in paste viscosity due to the high surface area and morphology of the HAP powder. An increase in viscosity in HAP slurries with HAP powders of increasing surface area was seen by F. Lelièvre, et al[16]. In addition, the acicular shape makes it increasingly difficult for the

particles to orient in the direction of shear due to increased particle - particle interactions. This reduction in particle mobility can result in increased viscosity values.

The stimuli –sensitive geling mechanism of the low *in vitro* viscosities calcium-phosphate composites described here are in contrast with earlier described composites[10,11,15]. The injectable calcium phosphate composites described in the literature may be divided in two groups: 1) self-hardening calcium phosphate pastes and cements, and 2) polymer/ceramic composites in which the ceramic phase is held in place by a highly viscous polymeric component. [12-14]. The composites in the first group undergo gelation within a few minutes after mixing where hardening is due to reactions in the ceramic phase, [10,11,1] which may cause handling problems [5]. The composites from the second group, run the risk of being washed away from the implantation site due to the "dilution" of the polymer component. The chitosan-calcium phosphate composites described in this paper may overcome both difficulties due to the fact that they do not gel upon mixing but, rather, gel *in vivo* in response to physiological stimulus.

Water content in cured composites

In these studies, chitosan-calcium phosphate pastes were cured in PBS solutions at pH 7.4. When exposed to physiological pH, the paste-like chitosan-calcium phosphate suspensions changed into solid polymer-ceramic composites with the ceramic components entrapped effectively within the gel matrix. This was due to the fact that the chitosan geling transition takes place around pH 6.5 resulting in formation of a polymeric gel network at physiological pH. It is known that geling transitions in polymeric solution

may result in volume changes due to the exclusion of water (syneresis). For the bone filler applications, it is desirable that gelation of the chitosan/calcium phosphate composite in a bone defect occur with no significant volume change. To evaluate possible volume changes that may occur due to the geling transition, the initial water content in chitosan/calcium phosphate suspensions (uncured pastes) was compared with the water content of the cured composites.

The water content was determined for the cured chitosan-HAP composites containing HMW chitosan with 30, 40 and 45 wt.% of HAP. The results presented in Figure 4 indicate that amount of water in the composite remained practically unchanged (within 4%) after the geling transition of chitosan. Thus, chitosan-HAP composites maintained a high water content in the cured state which may facilitate transport of nutrients and promote cell proliferation. In addition, for proper void filling capability, it is vital that the composites maintain contact with the surrounding bone and not shrink into a mass that may potentially migrate away from the wound site.

Microstructure of chitosan-HAP composites

It is well established that the porosity of a bone scaffold can have a dramatic effect on bone integration (REF). The importance of porous structure in promoting cell anchoring and proliferation, thus faster bone regeneration, has been already demonstrated by numerous studies on preformed scaffolds serving as bone substitutes. Pores that are too small will not allow osteoblast cell penetration into the network, thus leading to a

decrease in integration. Therefore it is important, when developing new formulations, to maintain adequate pore size to allow for osteoblast pentration.

The effect of the amount of ceramic present in the composite on the porosity and pore size was investigated in freeze-dried composites using field emission SEM. Figure 5A illustrates a relatively compact structure of the freeze-dried chitosan-HAP composite containing 40 wt% of the ceramic phase. As expected, when the solid content is reduced, as in a 20 wt % of HAP composite, a higher porosity as well as larger pores were obtained. Thus, composites that have a solids loading that is too large, may prevent the desired osteointergration due to pores sizes that are smaller than the osteoblast cell. (Figure 5B). For this work, it was determined that polymer ceramic composites in the range of 25 wt% solids content produced 3 dimensional structures with the desired pore size.

Additionally, higher magnification of these composite clearly illustrates the composite's 3 dimensional structure and illustrate that the polymer strands bind together the small particles of the ceramic phase (Fig 5C).

Compressive modulus

Compressive modulus is a measure of the resistance of the sample to compression and is an indicator of stiffness or rigidity. Relative compression moduli were determined to assess the changes in mechanical properties of the polymer-ceramic composites resulting from gelation of the polymeric component. The compressive moduli were determined

for a series of polymer-ceramic composites differing in composition in terms of ceramic (HAP or TCP) as well as polymeric components (low molecular weight (LMW) or high molecular weight (HMW)θ chitosan). Results are summarized in Table 3. Composites containing HAP exhibited higher relative compressive moduli than composites containing the same amount of TCP. (Compare, for example, the LMW/HAP 30 and LMW TCP 30 in Table 3) The trend was observed for both HMW and LMW chitosans. The higher relative compressive moduli of the composites containing HAP may be explained by differences in ceramic phase and morphology between HAP and TCP. As described above, the HAP powder consisted of loosely structured agglomerates with a rough, whisker-like surface (Fig.2) and α-TCP consisted of compact, smooth-surfaced primary particles ranging in size from 0.5-1.0 μm, and some 1-4 μm agglomerates (Fig. 3). It is believed that the whisker-like morphology of HAP particles contributed, through a steric hindrance effect, to the observed higher compressive strength of the chitosan-HAP composites.

As intuitively expected, the composites with higher solids loading, 30 vs. 20 wt.% of HAP, demonstrated higher relative compressive modulus (Compare, for example, HMW/HAP 20 with HMW/HAP 30 in Table 3)

The composite material before gelation is in essence a paste or a highly loaded polymeric body. Upon introduction of shear, the ceramic particles are able to move and rearrange themselves within the polymeric matrix. Upon gelation, however, the ceramic particles are unable to migrate within the polymeric phase. They are trapped within the chains of the chitosan. The compressive strength is a measure of a materials ability to

withstand crushing loads. If the ceramic is able to move relatively freely within the polymeric matrix, the relative compressive modulus of the material should be low. However, if the ceramic particles are trapped and unable to move about, the relative compressive modulus of the material should increase. Overall, the compressive modulus studies demonstrated this behavior in that the gelling transition changed the compressive modulus of chitosan/calcium phosphate composites. All of the studied composites turned from flowable suspensions (Fig.1) into solids with relative compressive strengths (Table 2). The compressive modulus of all the investigated composites is substantially lower then this of a bone or bone substitute materials . However, the role of the investigated composites is to provide scaffold for a new bone formation, that would in time provide the healed defect with the necessary mechanical strength.

Enzymatic degradation

Another concern when developing a new bone filler materials is bioresorption rate. It is important that the material remain as a scaffold long enough for adequate bone regeneration. This is usually on the order of several weeks to a few months depending upon the defect size, patient health etc.

We have investigated the *in vitro* enzymatic degradation of chitosan-HAP composites in the presence of lysozyme in order to determine the point at which the polymer/calcium phosphate structure breaks down. This enzyme was chosen because soluble chitosan, it's films and crosslinked gels may be degraded by enzymatic hydrolysis catalyzed by lysozyme[17]. Lysozyme hydrolyzes (1-4) glycosidic linkages of chitin and certain

bacterial-wall peptidoglycans. It has also been shown that only chitin and partially deacetylated chitin are good substrates for lysozyme, while completely deacetylated chitin is not degradable. [18] Tomihata et al. [19] demonstrated that chitosan films that were deacetylated more then 73.3 mol% showed much slower degradation in vivo then the lower deacetylated chitosan derivatives and chitin itself. The 69 % deacetylated chitosan and chitin films implanted subdermally in rats lost 50 % of their weight within the first three weeks, whereas, the 73.3 % deacetylated chitosan lost barely 5% of its weight during the same time. Based on these results one may expect that the degree of deacetylation may be used as a parameter controlling the degradation rate of chitosan-ceramic composites. K.M. Varum, et al. [20] investigated *in vitro* degradation rates of partially N-acetylated chitosans in human serum. Their results indicated that chitosans are *mainly depolymerized by lysozyme and not by other enzymes and mechanisms. The recent in* vivo results by R.A. Muzarelli, on human enzymatic activities related to the therapeutic administration of chitin derivatives also demonstrated susceptibility to enzymatic depolymerization by lysozyme. [21]

Our preliminary degradation results indicated that about 50% of the composite (TCP or HAP) containing LMW chitosan and about 65% of the composite containing HMW chitosan degraded within the first five weeks, as measured by the sample weight change (Figure 8). Slight differences in degradation rate may be accounted for by differences in deacetylation degree of the two chitosan samples, with HMW chitosan (86.2% deacetylation) degrading faster than LMW chitosan (89.7% deacetylation). This rate of *in vitro* degradation suggests that, *in vivo*, our composites may provide sufficient

structural support for the regenerating bone. Future *in vivo* studies are necessary to determine the optimal resorption rates of the chitosan/calcium phosphate composites.

Release of therapeutic compounds

A growing trend in tissue scaffolds is that the materials must not only provide the physical matrix for tissue regeneration but should also provide therapeutic agents to facilitate healing. Examples of therapeutic agents that are often needed in treatment of bone defects include: antibiotics for the local treatment of possible infections, anticancer agents for the site-specific treatment of bone tumors, and osteoconductive agents such as growth factors and bone morphogenic proteins for enhancement of bone tissue regeneration. In order to asses our geling polymer-ceramic composites as delivery vehicles for therapeutic agents we have investigated the release of 5-fluorouracil (5-FU) a model anticancer agent, and bovine serum albumin (BSA) as a model protein.

The results of the release experiments are presented in Figures 9 and 10. As shown in Figure 9, the release kinetics of 5-FU from chitosan-calcium phosphate composites was not affected by the ceramic phase kind, i.e., HAP vs. TCP (both composites contained 40 wt % of calcium phosphate). In both cases almost 90 % of drug was released within 10 hours. As illustrated in Figure 10 the release BSA demonstrated different kinetics. While 5-FU released from the chitosan-TCP composite within 10 hours, BSA release was much slower and lasted for 40 hours. These results may be explained by the differences in the effective size of the releasing molecules as well as the affinity of the agent for the ceramic phase. The slower release kinetics of the BSA, a 60,000 g mol^{-1} macromolecule,

may be due to a lower effective diffusion coefficient within the polymer-ceramic matrix. Additionally, BSA is know to strongly and irreversibly bind to calcium phosphate ceramics (Refs). The release may therefore be related to the dissolution kinetics of the ceramic material. The smaller 5FU molecule(g mol^{-1}) may possess a high diffusion coefficient with in the matrix as well as exhibit a more reversible adsorption behavior with the ceramic particles, which is often the case with small molecules (ref).

CONCLUSIONS

The chitosan/calcium phosphate composites studied in this work offer a new approach to the development of bone fillers that set in response to physiological conditions but do not set in vitro, upon mixing of the components.

Work presented here show that the rheological properties of the chitosan/calcium phosphate composite suspensions could be optimized towards injectability. The water content and porosity of cured composites suggest suitability for cell anchoring and proliferation. Mechanical properties of the cured composites were optimized towards compressive strengths that may be sufficient for a bone filling scaffold in non-load bearing applications. Preliminary degradation studies indicated slow degradation rates that may be further optimized (by changing chitosan deacetylation degree) to allow for bone tissue ingrowth before a substantial loss of mechanical strength. Finally, feasibility of the release of therapeutic agents from the composites (including macromolecular compounds) was demonstrated.

Table 1

Biologically Relevant Calcium Phosphate Materials

Mineral	Formula	CaP/ ratio
Dicalcium phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	1.00
Octacalcium phosphate	$Ca_8H_2(PO_4)_6 \cdot 5H_2O)$	1.33
Tricalcium phosphate	$Ca_3(PO_4)_2$	1.50
Hydroxyapatite	$Ca_5(PO_4)_3OH$	1.67
Tetracalcium phosphate	$Ca_4P_2O_4$	2.00
Bioglass®		

Table 2

Summary of ~~Mechanical and~~ Microstructural Characteristics

Solid phase	Particle size(μm)	Specific surface area (m^2g^{-1})	wgt%	Pore size	~~compressive modus~~
HAP	1-5	61.2	40%		
HAP	1-5	61.2	20%		
TCP	0.5-1.0 1-4 (few)	1.529			
TCP	0.5-1.0 1-4 (few)	1.529			

Table 3

Compressive Modulus Data

Sample	Compressive Modulus [Pa]
HMW/HAP 20	$1.17x10^4 \pm 0.06 x10^4$
HMW/HAP 30	$3.48x10^4 \pm 0.17 x10^4$
LMW/HAP 30	$3.08x10^4 \pm 0.15 x10^4$
LMW/TCP 30	$1.48x10^4 \pm 0.07 x10^4$
HMW/TCP 30	$1.25x10^4 \pm 0.06 x10^4$

HMW-high molecular weight chitosan
LMW- low molecular weight chitosan
HAP- hydroxyapatite
TCP- tricalcium phosphate
HMW/HAP 20 – composite that contains high molecular weight chitosan with 20 wt% hydroxyapatite

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Figure Legends

Figure 1. A. Shear thinning behavior of chitosan/HAP composite containing 40 wt% HAP

B. Viscosity of chitosan/calcium phosphate, hydroxyapatite (HAP) or □-tricalcium phosphate (TCP) suspensions as a function of ceramic phase content. Reported viscosities were recorded at shear rate 116 1/s.

Figure 2. Scanning electron micrographs of hydroxyapatite powder. Magnification:

A. x4000, B. x 10,000

Figure 3. Scanning electron micrographs of □-tricalcium phosphate powder. Magnification:

A. x10,000, B. x 20,000

Figure 4. Water content in cured composites as a function of ceramic phase content

Figure 5. Field emission- scanning electron micrographs of chitosan- hydroxyapatite (HAP) composites containing: A. 40 wt.% HAP (magnification x1000), B. 20 wt.% HAP (magnification x1000), C. 20 wt.% HAP (magnification x10,000)

Figure 6. Relative compressive modulus of chitosan/calcium phosphate composites: Effect of ceramic phase solids loading

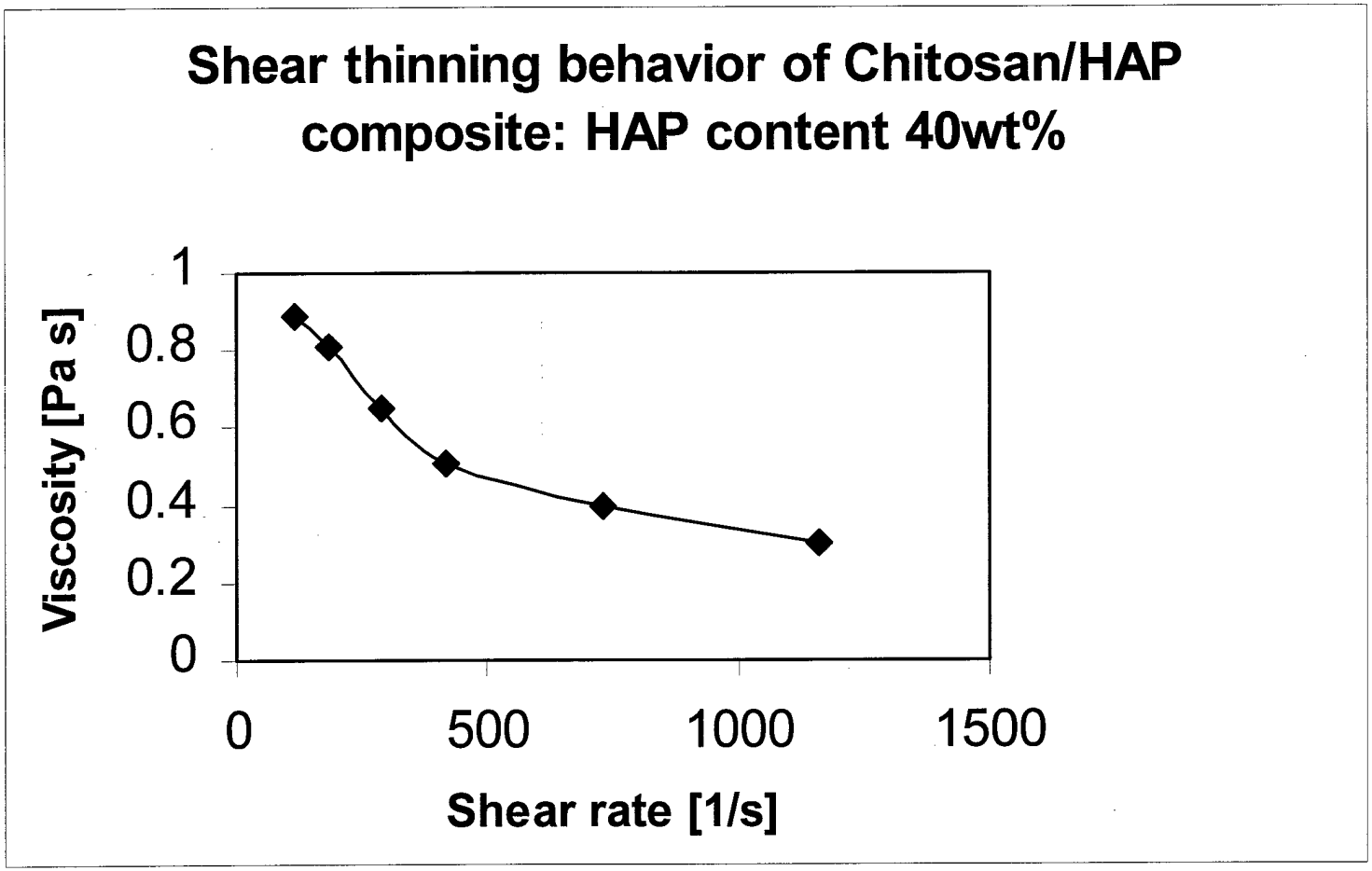
Figure 7. Relative compressive modulus of chitosan/calcium phosphate composites: Effect of the ceramic phase type (HAP vs. TCP) in composites with: A. high molecular weight (HMW) chitosan, B. low molecular weight (LMW) chitosan

Figure 8. In vitro enzymatic degradation of chitosan/HAP composites in phosphate buffered saline solutions containing 1mg/ml of lysozyme.

Figure 9. Release kinetics of 5-fluorouracil from chitosan/calcium phosphate composites: Effect of the ceramic phase kind (HAP vs. TCP). Both composites contained 40 wt % of the ceramic phase.

Figure 10. Release kinetics of 5-fluorouracil and bovine serum albumin from chitosan/TCP composites: molecular weight effect of the releasing agent (5-FU vs. BSA)

Figure 1


Shear thinning behavior of Chitosan/HAP composite: HAP content 40wt%
Viscosity [Pa s]
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0.8
0.6
0.4
0.2
0
0
500
1000
1500
Shear rate [1/s]

Figure 2

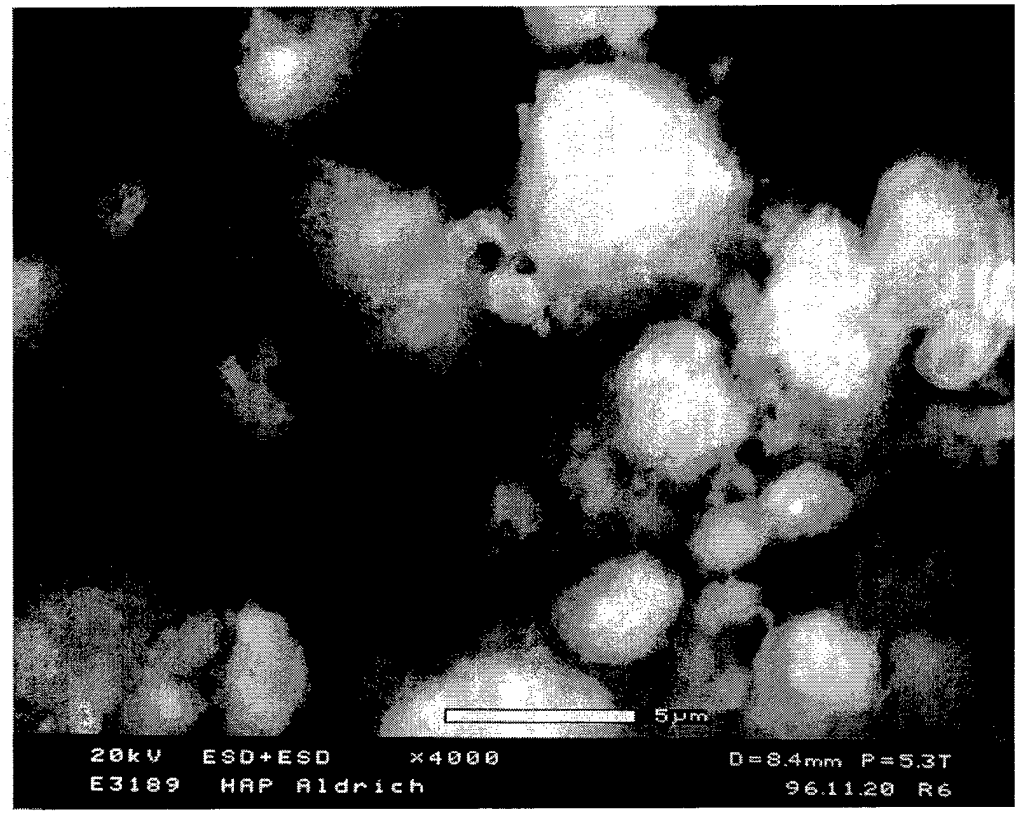

Figure 3

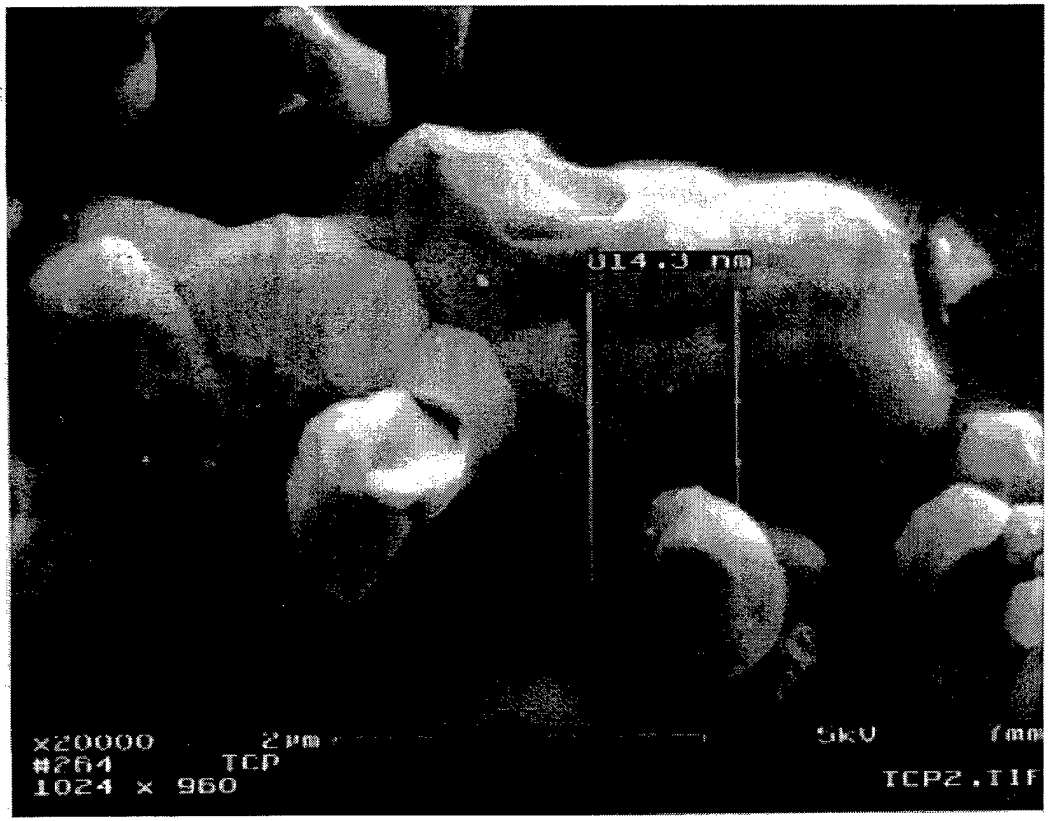

Figure 4

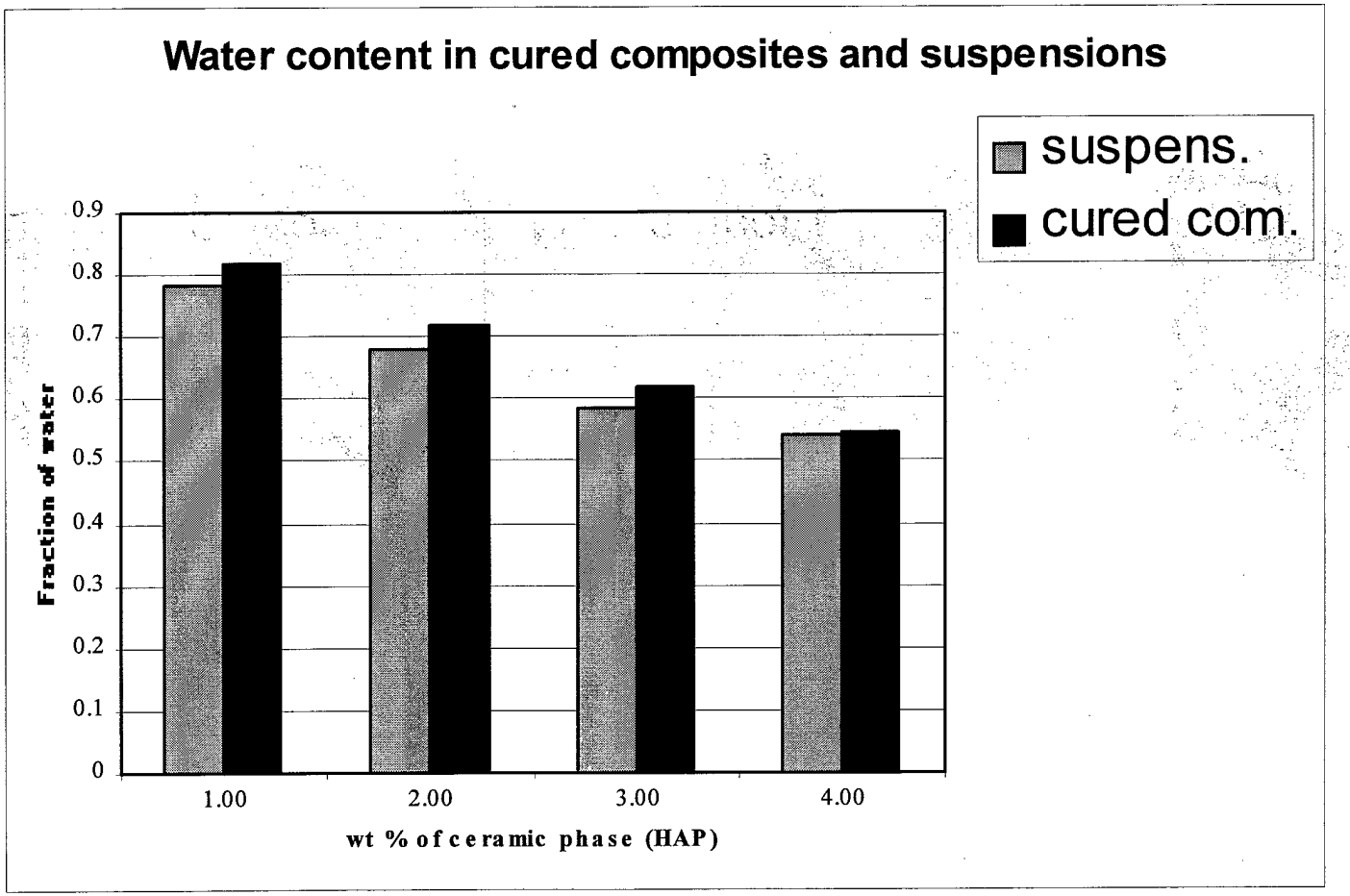
Water content in cured composites and suspensions
suspens.
cured com.
Fraction of water
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.00
2.00
3.00
4.00
wt % of ceramic phase (HAP)

Figure 5

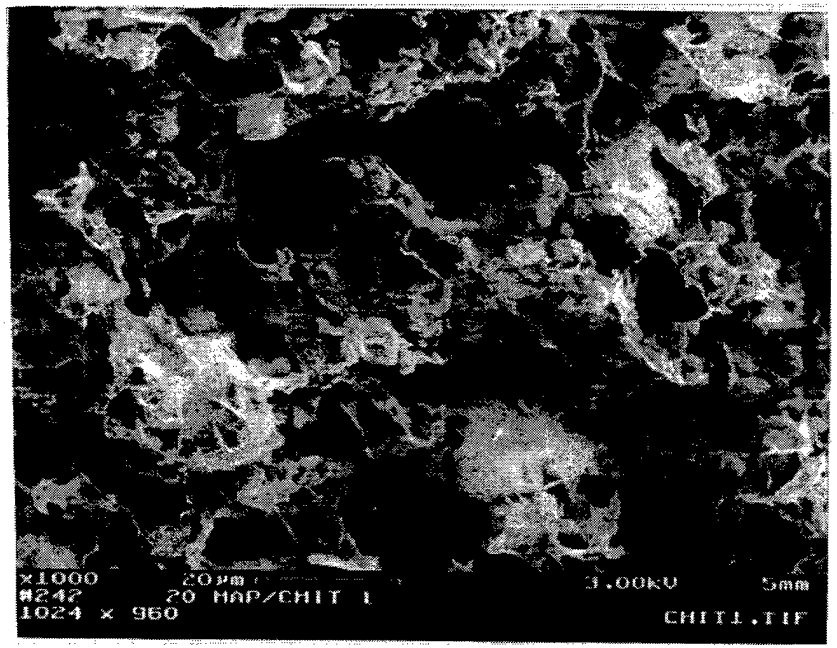

Figure 6

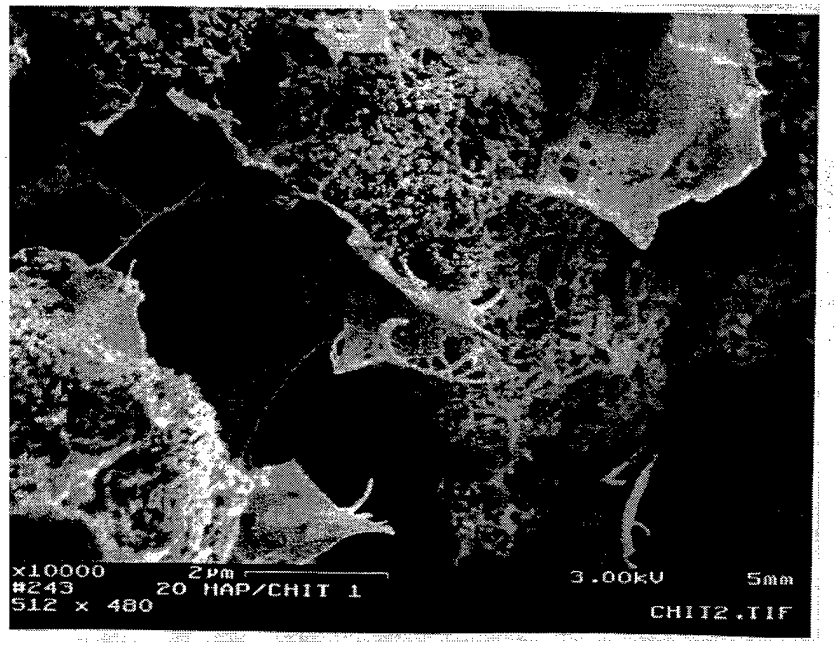

Figure 7

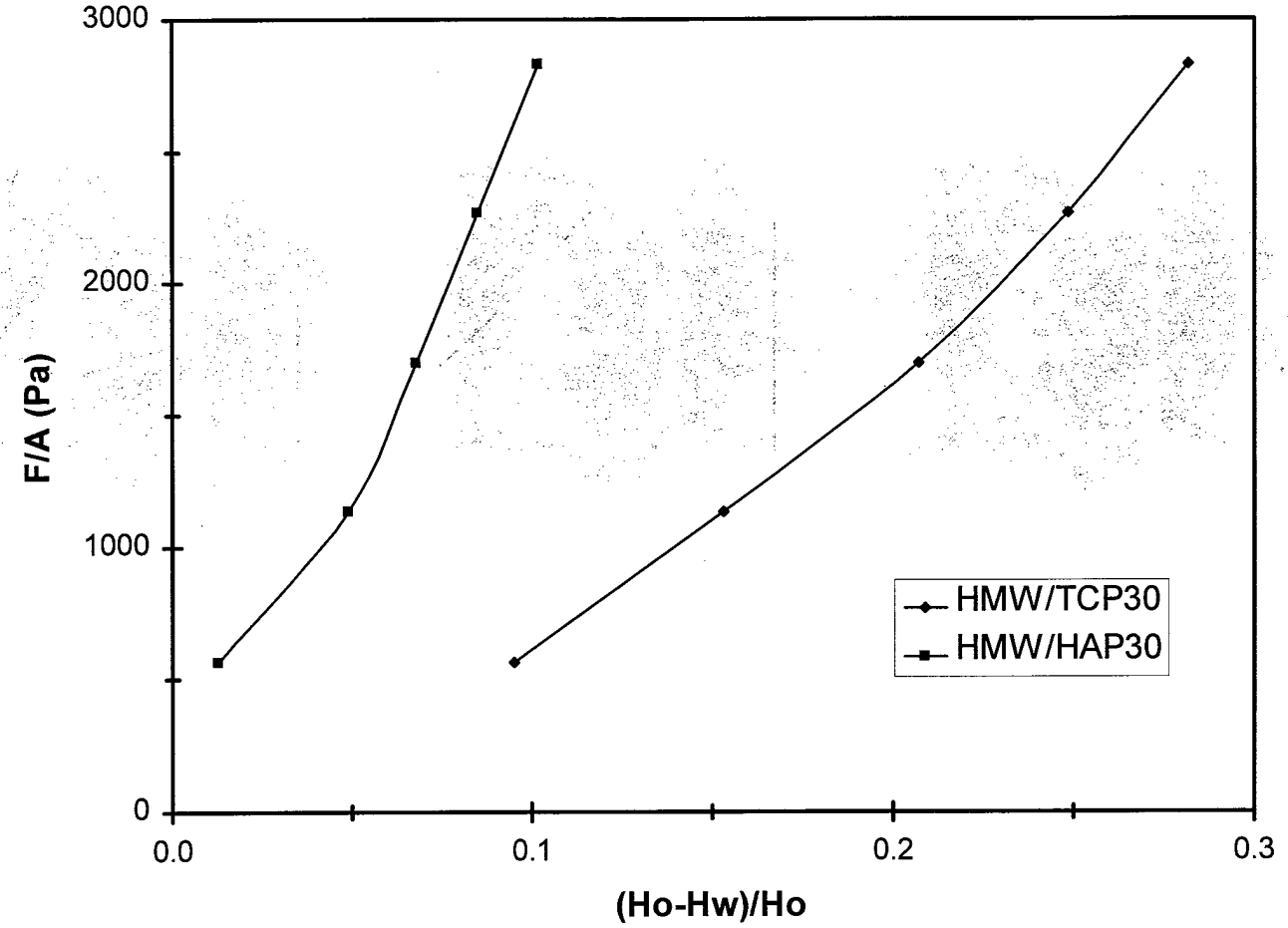

Figure 8